ANNEXURE 1

Trease and Evans' Pharmacognosy

FOURTEENTH EDITION

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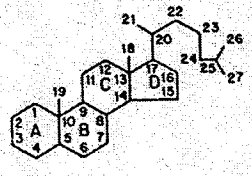
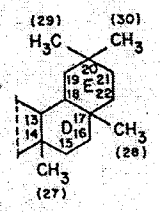
22 Saponins, Cardioactive Drugs and Other Steroids

Plant materials containing saponins have long been used in many parts of the world for their detergent properties. For example, in Europe the root of *Saponaria officinalis* (Caryophyllaceae) and in South America the bark of *Quillaia saponaria* (Rosaceae). Such plants contain a high percentage of glycosides known as saponins (Latin *sapo*, soap) which are characterized by their property of producing a frothing aqueous solution. They also have haemolytic properties, and when injected into the blood stream, are highly toxic. The fact that a plant contains haemolytic substances is not proof that it contains saponins, and in the species examined by Wall (1961) only about half of those containing haemolytic substances actually contained saponins. When taken by mouth, saponins are comparatively harmless. Sarsaparilla, for example, is rich in saponins but is widely used in the preparation of nonalcoholic beverages.

Saponins have a high molecular weight and a high polarity and their isolation in a state of purity presents some difficulties. Often they occur as complex mixtures with the components differing only slightly from one another in the nature of the sugars present, or in the structure of the aglycone. Various chromatographic techniques have been employed for their isolation. As glycosides they are hydrolysed by acids to give an aglycone (sapogenin) and various sugars and related uronic acids. According to the structure of the aglycone or sapogenin, two kinds of saponin are recognized—the steroidal (commonly tetracyclic triterpenoids) and the pentacyclic triterpenoid types (see formulae below). Both of these have a glycosidal linkage at C-3 and have a common biogenetic origin via mevalonic acid and isoprenoid units.



Steroid skeleton



Pentacyclic triterpenoid skeleton

A distinct subgroup of the steroidal saponins is that of the steroidal alkaloids which characterize many members of the Solanaceae. They possess a heterocyclic nitrogen-containing ring, giving the compounds basic properties (as an example see solasodine, Fig. 22.5).

STEROIDAL SAPONINS

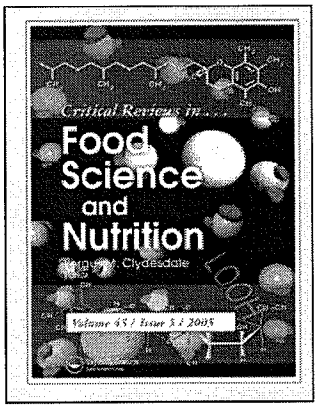
The steroidal saponins are less widely distributed in nature than the pentacyclic triterpenoid type. Phytochemical surveys have shown their presence in many monocotyledonous families, particularly the Dioscoreaceae (e.g. *Dioscorea* spp.), Amaryllidaceae (e.g. *Agave* spp.) and Liliaceae (e.g. *Yucca* and *Trillium* spp.). In the dicotyledons the occurrence of diosgenin in fenugreek (Leguminosae) and of steroidal alkaloids in *Solanum* (Solanaceae) is of potential importance. Some species of *Strophanthus* and *Digitalis* contain both steroidal saponins and cardiac glycosides (q.v.). Examples of saponins and their constituent sugars are given in Table 22.1.

Steroidal saponins are of great pharmaceutical importance because of their relationship to compounds such as the sex hormones, cortisone, diuretic steroids, vitamin D and the cardiac glycosides. Some are used as starting

Table 22.1. Examples of steroidal saponins.

Steroidal saponin	*Sugar components*	*Occurrence*
Sarsaponin (Parillin)	3 glucose, 1 rhamnose	*Smilax* spp.
Digitonin	2 glucose, 2 galactose, 1 xylose	Seeds of *Digitalis purpurea* and *D. lanata*
Gitonin	1 glucose, 2 galactose, 1 xylose	Seeds and leaves of *D. purpurea* and seeds of *D. lanata*
Dioscin	1 glucose, 2 rhamnose	*Dioscorea* spp.

ANNEXURE 2



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Saponins: Properties, Applications and Processing

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Saponins: Properties, Applications and Processing

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Saponins are a diverse group of compounds widely distributed in the plant kingdom, which are characterized by their structure containing a triterpene or steroid aglycone and one or more sugar chains. Consumer demand for natural products coupled with their physicochemical (surfactant) properties and mounting evidence on their biological activity (such as anticancer and anticholesterol activity) has led to the emergence of saponins as commercially significant compounds with expanding applications in food, cosmetics, and pharmaceutical sectors. The realization of their full commercial potential requires development of new processes/processing strategies to address the processing challenges posed by their complex nature. This review provides an update on the sources, properties, and applications of saponins with special focus on their extraction and purification. Also reviewed is the recent literature on the effect of processing on saponin structure/properties and the extraction and purification of sapogenins.

Keywords Triterpenes, sapogenins, ginsenosides, health products, surfactants, extraction

INTRODUCTION

Saponins, glycosides widely distributed in the plant kingdom, include a diverse group of compounds characterized by their structure containing a steroidal or triterpenoid aglycone and one or more sugar chains. Their structural diversity is reflected in their physicochemical and biological properties, which are exploited in a number of traditional (as soaps, fish poison, and molluscicides) and industrial applications (Price et al., 1987; Oakenfull, 1981; Fenwick et al., 1991; Hostettmann and Marston, 1995; Oakenfull and Sidhu, 1989). While plant extracts containing saponins have been widely used in food and other industrial applications mainly as surface active and foaming agents (San Martin and Briones, 1999); saponins in foods have traditionally been considered as "antinutritional factors" (Thompson, 1993) and in some cases have limited their use due to their bitter taste (Ridout et al., 1991). Therefore, most of the earlier research on processing of saponins targeted their removal to facilitate human consumption (Khokhar and Chauhan, 1986; Ridout et al., 1991). However, food and non-food sources of saponins have come into renewed focus in recent years due to increasing evidence of their health benefits such as cholesterol lowering and anticancer properties (Gurfinkel and Rao, 2003; Kim et al., 2003b). Recent research has established saponins as the active components in many herbal medicines (Liu and Henkel, 2002; Alice et al., 1991) and highlighted their contributions to the health benefits of foods such as soybeans (Kerwin, 2004; Oakenfull, 2001) and garlic (Matsuura, 2001).

The commercial potential of saponins has resulted in the development of new processes/processing strategies and reevaluation of existing technologies (Muir et al., 2002) for their extraction/concentration (Rickert et al., 2004b). The objective of this review is to provide a timely update on the sources, properties and applications of saponins with special focus on their extraction and purification.

SOURCES

The presence of saponins has been reported in more than 100 families of plants, and in a few marine sources such as star fish and sea cucumber (Hostettmann and Marston, 1995). The steroidal saponins are mainly found in monocotyledons (such as *Agavaceae, Dioscoreaceae* and *Liliaceae*), and triterpene saponins are predominantly present in dicotyledons (*Leguminosae, Araliaceae, Caryophyllaceae*) (Sparg et al., 2004). While the main dietary sources of saponins are legumes (soybeans,

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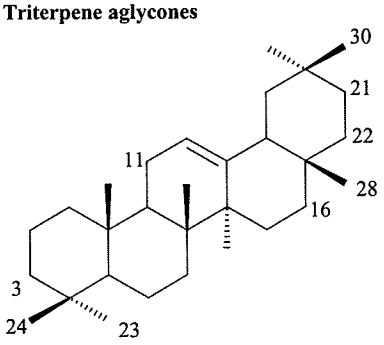
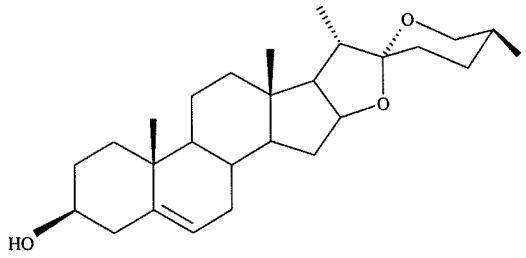
(A) AGLYCONES

Triterpene aglycones



Aglycone	-OH	=O	-COOH
Glycyrrhetinic acid	3β	11	30
Gypsogenin	3β	23	28
Oleanolic acid	3β		28
Quillaic acid	3β, 16α	23	28
Soyasapogenol A	3β, 21β, 22β, 24		
Soyasapogenol B	3β, 22β, 24		
Soyasapogenol E	3β, 24	22	

Steroid aglycone
Diosgenin



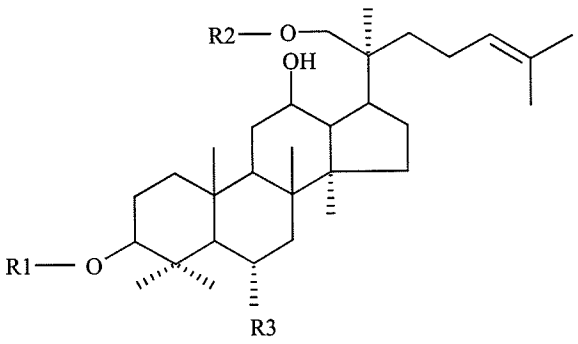
(B) SOYASAPONINS

Soyasaponin	Soyasapogenol	Structure[a]
Group A		
Aa	A	glc(1→2)gal(1→2)glcUA(1→3)**A**(22←1)ara(3←1)xyl(2,3,4-tri-*O*-Acetyl)
Ab	A	glc(1→2)gal(1→2)glcUA(1→3) **A**(22←1)ara(3←1) glc(2,3,4,6-tetra-*O*-Acetyl)
Ac	A	rha(1→2)gal(1→2)glcUA(1→3) **A**(22←1)ara(3←1) glc(2,3,4,6-tetra-*O*-Acetyl)
Ad	A	glc(1→2)ara(1→2)glcUA(1→3) **A**(22←1)ara(3←1) glc(2,3,4,6-tetra-*O*-Acetyl)
Ae	A	gal(1→2)glcUA(1→3) **A**(22←1)ara(3←1)xyl(2,3,4-tri-*O*-Acetyl)
Af	A	gal(1→2)glcUA(1→3) **A**(22←1)ara(3←1) glc(2,3,4,6-tetra-*O*-Acetyl)
Ag	A	ara(1→2)glcUA(1→3) **A**(22←1)ara(3←1)xyl(2,3,4-tri-*O*-Acetyl)
Ah	A	ara(1→2)glcUA(1→3) **A**(22←1)ara(3←1) glc(2,3,4,6-tetra-*O*-Acetyl)
Group B		
Ba	B	glc(1→2)gal(1→2)glcUA(1→3)**B**
Bb	B	rha(1→2)gal(1→2)glcUA(1→3)**B**
Bc	B	rha(1→2)ara(1→2)glcUA(1→3)**B**
Bb'	B	gal(1→2)glcUA(1→3)**B**
Bc'	B	ara(1→2)glcUA(1→3)**B**
Group E		
Bd	E	glc(1→2)gal(1→2)glcUA(1→3)**E**
Be	E	rha(1→2)gal(1→2)glcUA(1→3)**E**
DDMP		
αg	B_{DDMP}[b]	glc(1→2)gal(1→2)glcUA(1→3)$\mathbf{B}_{DDMP}$
βg	B_{DDMP}[b]	rha(1→2)gal(1→2)glcUA(1→3)$\mathbf{B}_{DDMP}$
βa	B_{DDMP}[b]	rha(1→2)ara(1→2)glcUA(1→3)$\mathbf{B}_{DDMP}$
γg	B_{DDMP}[b]	gal(1→2)glcUA(1→3)$\mathbf{B}_{DDMP}$
γa	B_{DDMP}[b]	ara(1→2)glcUA(1→3)$\mathbf{B}_{DDMP}$

[a] glc:D-glucose, ara:L-arabinose, gal:D-galactose, glcUA:D-glucuronic acid, xyl:D-xylose, rha: L-rhamnose
[b] B_{DDMP}: DDMP (2,3-dihydro-2,5-dihydroxy-6-methyl-4H-pyran-4-one) attached through an acetal linkage to the C-22 hydroxyl of soyasapogenol B

Figure 1 Structure of (A) aglycones (Hostettman and Marston, 1995), (B) soyasaponins (Berhow et al., 2002; Gu et al., 2002), (C) ginsenosides (Li et al., 1996), (D) glycyrrhizic acid (Ong and Len, 2003), and (E) quillaja saponins (Reprinted from Nord and Kenne, 2000, Copyright (2002) with permission from Elsevier). (Continued)

(C) GINSENOSIDES



Ginsenosides	R1	R2	R3
Rb_1	-glc[2→1]glc	-glc[6→1]glc	-H
Rb_2	-glc[2→1]glc	-glc[6→1]ara(p)	-H
Rc	-glc[2→1]glc	-glc[6→1]ara(f)	-H
Rd	-glc[2→1]glc	-glc	-H
Re	-H	-glc	-O-glc[2→1]rha
Rf	-H	-H	-O-glc[2→1]glc
Rg_1	-H	-glc	-O-glc

glc: D-glucose, ara(p): L-arabinopyranose, ara(f): L-arabinofuranose, rha: L-rhamnose

(D) GLYCYRRHIZIC ACID (GLYCYRRHIZIN)

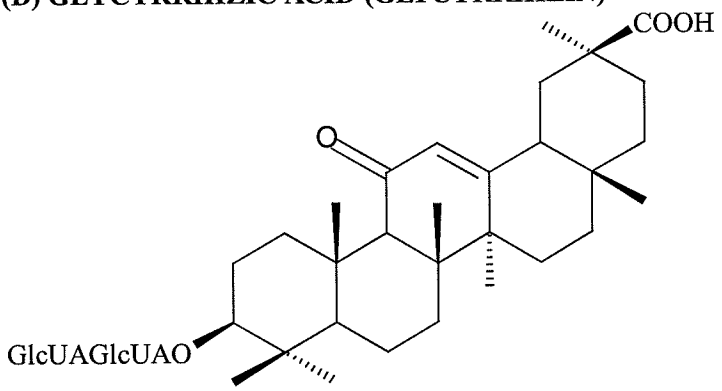


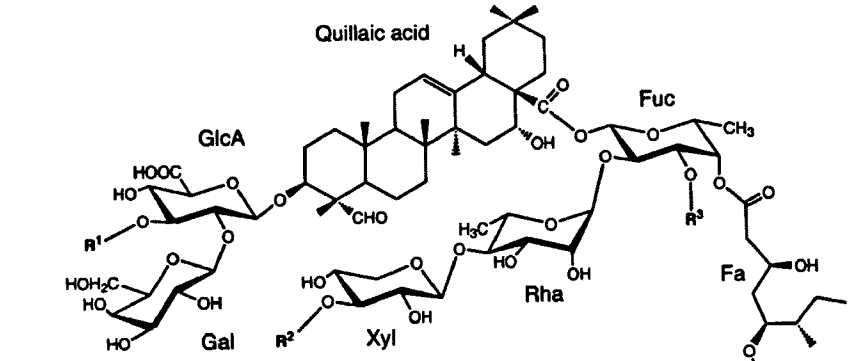
Figure 1 (Continued)

chickpeas, mungbeans, peanuts, broad beans, kidney beans, lentils), they are also present in oats, allium species (leek, garlic), asparagus, tea, spinach, sugarbeet, and yam (Price et al., 1987). Soap bark tree (*Quillaja saponaria*), fenugreek (*Trigonella foenum-graceum*), alfalfa (*Medicago sativa*), horse chestnut (*Aesculus hippocastanum*), licorice (Glycyrrhiza species such as *Glycyrrhiza glabra*), soapwort (*Saponaria officinalis*), Mojave yucca (*Yucca schidigera*), gypsophila genus (such as *Gypsophila paniculata*), sarsaparilla (*Smilax regelii* and other closely related species of Smilax genus) and ginseng (Panax genus) are the main non-food sources of saponins used in health and industrial applications (Hostettmann and Marston, 1995; Balandrin, 1996).

A single plant species may contain a complex mixture of saponins. For example, the characterized soybean saponins include three groups of compounds: soyasaponins A, B and E categorized according to the soyasapogenol in their structure (Figure 1B). Similarly ginseng contains a mixture of saponins (ginsenosides), the main components of which are Rb_1, Rb_2, Rc, Rd, Re, Rf, and Rg_1 (Figure 1C). Commonly used plant sources and their main saponins are presented in Table 1.

The saponin content of plant materials is affected by the plant species, genetic origin, the part of the plant being examined, the environmental and agronomic factors associated with growth of the plant, and post-harvest treatments such as storage and processing (Fenwick et al., 1991) (Table 2).

(E) QUILLAJA SAPONINS



	R^1	R^2	R^3
S1	α-L-Rha*p*	H	H
S2	β-D-Xyl*p*	H	H
S3	α-L-Rha*p*	β-D-Xyl*p*	H
S4	β-D-Xyl*p*	β-D-Xyl*p*	H
S5	α-L-Rha*p*	β-D-Api*f*	H
S6	β-D-Xyl*p*	β-D-Api*f*	H
S7	α-L-Rha*p*	H	Acetyl
S8	β-D-Xyl*p*	H	Acetyl
S9	α-L-Rha*p*	β-D-Xyl*p*	Acetyl
S10	β-D-Xyl*p*	β-D-Xyl*p*	Acetyl
S11	α-L-Rha*p*	β-D-Api*f*	Acetyl
S12	β-D-Xyl*p*	β-D-Api*f*	Acetyl

Figure 1 (Continued)

STRUCTURE AND PROPERTIES

Structure

Saponins are glycosides containing one or more sugar chains on a triterpene or steroid aglycone backbone also called a sapogenin (Figure 1). They are categorized according to the number of sugar chains in their structure as mono, di-, or tridesmosidic. Monodesmosidic saponins have a single sugar chain, normally attached at C-3. Bidesmosidic saponins have two sugar chains, often with one attached through an ether linkage at C-3 and one attached through an ester linkage at C-28 (triterpene saponins) or an ether linkage at C-26 (furastanol saponins). The most common monosaccharides include: D-glucose (Glc), D-galactose (Gal), D-glucuronic acid (GlcA), D-galacturonic acid (GalA), L-rhamnose (Rha), L-arabinose (Ara), D-xylose (Xyl), and D-fucose (Fuc). The nature of the aglycone and the functional groups on the aglycone backbone and number and nature of the sugars can vary greatly resulting in a very diverse group of compounds (Figure 1; Price et al., 1987; Hostettmann and Marston, 1995).

Properties

Physicochemical Properties

The structural complexity of saponins results in a number of physical, chemical, and biological properties, only a few of which are common to all members of this diverse group. Properties of a few selected aglycones and saponins are summarized in Table 3.

Due to the presence of a lipid-soluble aglycone and water-soluble sugar chain(s) in their structure (amphiphilic nature), saponins are surface active compounds with detergent, wetting, emulsifying, and foaming properties (Wang et al., 2005; Sarnthein-Graf and La Mesa, 2004; Mitra and Dungan, 1997; Ibanoglu and Ibanoglu, 2000). In aqueous solutions surfactants form micelles above a critical concentration called critical micelle concentration (cmc). Saponins, including soybean saponins, saponins from *Saponaria officinalis*, and *Quillaja saponaria*, form micelles in aqueous solutions, the size and structure of which are dependent on type of saponin (Oakenfull, 1986). The micelle forming properties (cmc and the aggregation number (number of monomers in a micelle)) of quillaja

Table 1 Selected plant sources and their constituent saponins

Source	Aglycone	Saponin	Reference
Soybean	Soyasapogenol A	Acetyl soyasaponins A_1 (Ab), A_2 (Af), A_3, A_4 (Aa), A_5 (Ae), A_6, A_c, A_d	Yoshiki et al., 1998
	Soyasapogenol B	Soyasaponin DDMP[a] conjugated I (Bb) βg II (Bc) βa III (Bb′) γg IV (Bc′) γa V (Ba) αg	Yoshiki et al., 1998
	Soyasapogenol E	Soyasaponin Be, Bd	Yoshiki et al., 1998
Chickpea	Soyasapogenol B	DDMP[a] conjugated saponins	Kerem et al., 2005; Price et al., 1988
Quillaja	Quillaic acid	QS 1-22, S1-12	Kensil and Marciani, 1991; Nord and Kenne, 2000
Horse chestnut	Protoescigenin, barringtogenol C	Aescin (escin): β-aescin, cryptoaescine, α-aescine	World Health Organization, 2001
Alfalfa	Medicagenic acid	I-XV	Oleszek, 1995
	Hederagenin	XVI-XIX	Oleszek, 1995
	Soyasapogenol B, E	XX- XXVI	Oleszek, 1995
	Zanhic acid	XXV-XXVI	Oleszek, 1995
Licorice	Glycyrrhetic acid	Glycyrrhizic acid[b]	World Health Organization, 1999a
Ginseng	20(s)-protopanaxadiol	Ra_{1-3}, Rb_{1-3}, Rc, Rc_2, Rd, Rd_2, Rh_2	World Health Organization, 1999b
	20(s)-protopanaxatriol	Re_2, Re_3, Rf, Rg_1, Rg_2, Rh_1	World Health Organization, 1999b
Quinoa	Phytolaccagenic acid Oleanolic acid Hederagenin	Quinoa saponins	Mizui et al., 1990
Oat	Nuategenin	Avenacoside A, B	Önning et al., 1994
Yam (Dioscoera species)	Diosgenin	Dioscin	Hostettmann and Marston, 1995
Fenugreek	Diosgenin, yamogenin, tigogenin, neotigogenin, yuccagenin, lilagenin, gitogenin, neogitogenin, smilagenin, sarsasapoenin	Trigofoenoside A-G, Trigonelloside B (C)	Sauvaire et al., 1995

[a]2,3-dihydro-2,5-dihydroxy-6-methyl-4*H*-pyran-4-one.
[b]Synonyms: glycyrrhizin, glycyrrhizinic acid.

saponins were affected by temperature, salt concentration, and pH of the aqueous phase (Mitra and Dungan, 1997). At 25°C, the values of cmc of quillaja saponins were in the range of 0.5 and 0.8 g/L. It increased with temperature and pH but decreased with increasing salt concentration. The incorporation of cholesterol into the saponin micelles increased their cmc, size, viscosity, and the aggregation number (Mitra and Dungan, 2000) resulting in the solubility enhancement of cholesterol as much as a factor of 10^3 at room temperature (Mitra and Dungan, 2001).

Table 2 Saponin content of some selected plant materials

Source	Saponin content (%)	Reference
Soybean	0.22–0.47	Fenwick et al., 1991
Chickpea	0.23	Fenwick et al., 1991
Green pea	0.18–4.2	Price et al., 1987
Quillaja bark	9–10	San Martin and Briones, 1999
Yucca	10	Oleszek et al., 2001
Fenugreek	4–6	Sauvaire et al., 2000
Alfalfa	0.14–1.71	Fenwick et al., 1991
Licorice root	22.2–32.3	Fenwick et al., 1991
American ginseng *(P. quinquefolium L).*		
Young leaves	1.42–2.64	Li et al., 1996
Mature leaves	4.14–5.58	Li et al., 1996
Roots (4 year old)	2.44–3.88	Li et al., 1996
Oat	0.1–0.13	Price et al., 1987
Horse chest nut	3–6	Price et al., 1987
Sugar beet leaves	5.8	Price et al., 1987
Quinoa	0.14–2.3	Fenwick et al., 1991

Quillaja saponins also had a solubilizing effect on phenantherene, and fluoranthene, which increases linearly with saponin concentration at values higher than cmc (Soeder et al., 1996). A similar linear relationship has been observed between the concentration of the saponin extract from *Sapindus mukurossi* and aqueous solubility of hexachlorobenzene and naphthalene up to a surfactant concentration of 10% (Kommalapati et al., 1997; Roy et al., 1997).

Solubility enhancement has also been observed for Yellow OB (Nakayama et al., 1986), and progesterone (Nakayama et al., 1986) in the presence of bidesmoside saponins from *Sapindus mukurossi*, and for α-tocopherol, and oleanolic acid in the presence of glucoside and glucuronide esters of glycyrrhizic acid (Sasaki et al., 1988). Purified saponins and saponin mixtures resulted in both enhancements and reductions in water solubility of test compounds quercetin (Schöpke and Bartlakowski, 1997), digitoxin (Walthelm et al., 2001), rutin (Walthelm et al., 2001), and aesculin (Walthelm et al., 2001), the extent of which was determined by concentration of saponin and the model compound. Solubility enhancement of quercetin obtained by pure

Table 3 Physical properties of some selected aglycones and saponins (Adapted from Budavari et al., 1996; Biran and Baykut, 1975)

Compound	Formula	Solubility	Source	MW	MP
Aglycone					
Oleanolic acid	$C_{30}H_{48}O_3$	Insoluble in water, sol in 65 parts ether, 106 parts 95% alcohol, 35 parts boiling 95% alcohol, 118 parts chloroform, 180 parts acetone, 235 parts methanol.	Quinoa	457	310
Quillaic acid	$C_{30}H_{46}O_5$	Soluble in alcohol, ether, acetone, ethyl acetate, glacial acetic acid	Quillaja	487	292–293
Diosgenin	$C_{27}H_{42}O_3$	Soluble in the usual organic solvents, in acetic acid	Dioscorea, fenugreek, yam	415	204–207
Glycyrrhetic acid	$C_{30}H_{46}O_4$		Licorice	471	298–300
Saponin					
Glycyrrhizic acid (Glycyrrhizin)	$C_{42}H_{62}O_{16}$	Freely soluble in hot water, alcohol, practically insoluble in ether	Licorice	823	
Escin			Horse chestnut		
α-escin		Very soluble in water and methanol, only slightly soluble in acetone, insoluble in ether and hydrocarbons			225–227
β-escin		Readily soluble in methanol, slightly soluble in acetone, practically insoluble (very little solubility) in water, insoluble in ether and hydrocarbons			222–223
Gypsophia saponin	$C_{35}H_{61}O_{24}$	Soluble in water (0.5147 g/100 mL at 25°C)	Gypsophia	863	221–227

saponins at concentrations > cmc values can be attributed to micellar solubilization, whereas solubilization effect of some saponin mixtures at concentrations < cmc points to an alternative mechanism (Schöpke and Bartlakowski, 1997).

Purified saponins or saponin mixtures may also have a solubilizing effect on other saponins. Solubility enhancement of monodesmosides (such as monodesmosides of *Sapindus mukurossi* (Nakayama et al., 1986; Kimata et al., 1983), *Bupleuri radix* (saikosaponins) (Kimata et al., 1985; Morita et al., 1986; Watanabe et al., 1988) and soyasaponins Bb, Bb′ and B-G (Shimoyamada et al., 1993)), which have very low water solubility, in the presence of bidesmoside saponins is well documented. The extent of the enhancement is dependent on the structure of the monodesmoside saponin, and the composition/concentration of the saponin bidesmosides. Solubility of *Sapindus mukurossi* monodesmosides was enhanced in the presence of mukurossi bidesmoside saponins containing hederagenin (Y1, Y2, X) (Nakayama et al., 1986; Kimata et al., 1983). However, mukurossi bidesmosides did not affect the solubility of saikosaponins (Kimata et al., 1985), which was enhanced by oleanolic acid bidesmosides with a glucuronide moiety such as ginsenosides (chikusetsusaponin-V (ginsenoside Ro) and IV) (Kimata et al., 1985; Watanabe et al., 1988), *Hemsleya macrosperma* (cucurbitaceae) bidesmosides (Ma2 and Ma3) (Morita et al., 1986), and cyclic bidesmoside tubeimoside I isolated from tubers of *Bolbostemma paniculatum* Franquet (Kasai et al., 1986b).

The solubility of saikosaponin-a in water at 37°C (0.14 mg /mL) increased with concentration of ginsenosi de Ro reaching a value of 4.08 mg/mL at a bidesmoside concentration of 1.4 mg/mL (Kimata et al., 1985). A significant decrease in the solubilizing effect on saikosaponin-a was observed upon methylation or reduction of the glucuronide carboxyl group of ginsenoside-Ro indicating the role of the glucuronide moiety in the observed effect (Tanaka, 1987). A greater extent of enhancement was obtained for *Hemsleya macrosperma* (cucurbitaceae) bidesmosides Ma2 and Ma3, which are structurally similar to ginsenoside Ro with similar cmc values, at a concentration of 0.1% resulting in saikosaponin-a solubilities of 5–8.7 mg/mL compared to 3.4 mg/mL for ginsenoside Ro (Morita et al., 1986). The solubility enhancement of saikosaponin-a became apparent near the cmc of these bidesmosides (Kimata et al., 1985; Morita et al., 1986; Nakayama et al., 1986).

The solubility of diene saponin saikosaponin-b1 produced by heating or mild-acid treatment of saikosaponin-a was increased by malonyl-ginsenosides and to a lesser extent by ginsenoside Ro (Zhou et al., 1991). The effect of malonyl-ginsenosides on saikosaponin-a has also been demonstrated (Zhou et al., 1991). While neutral dammarane ginsenosides did not have a solubilizing effect on saikosaponins by themselves, they enhanced the solubilizing effect of ginsenoside Ro (Watanabe et al., 1988) and dammarane ginsenosides (Zhou et al., 1991).

Solubility enhancement of saikosaponin-a has also been observed in the presence of glycyrrhizic acid, which is the glucuronide monodesmoside saponin of licorice (Sasaki et al., 1988). The decrease in the degree of enhancement observed at high glycyrrhizic acid concentrations was attributed to the increase in solution viscosity (Sasaki et al., 1988). A solubilizing effect was also observed for the 30-β-glucoside (isolated

from licorice roots) and glucuronide esters of glycyrrhizic acid at higher concentrations (Sasaki et al., 1988). In addition to bidesmosides, co-occuring compounds such as acyclic sesquiterpene oligoglycosides have also been shown to have a solubilizing effect on monodesmosides of *Sapindus mukurossi* (Kasai et al., 1986a) and *Sapindus delavayi* (Wong et al., 1991).

Solubility enhancement may have important implications for the bioactivity and processing of saponins. Monodesmosides, while poorly soluble in water in purified form, can be extracted readily due to the solubilizing effect of co-occuring compounds (Kimata et al., 1983). Micellar solubilization by saponins can be exploited for the development of micellar extraction processes or to affect the solubilization of ingredients in cosmetic, pharmaceutical or food formulations (Shirakawa et al., 1986).

Solubility of saponins is also affected by the properties of the solvent (as affected by temperature, composition, and pH). While water, alcohols (methanol, ethanol) and aqueous alcohols are the most common extraction solvents for saponins, solubility of some saponins in ether, chloroform, benzene, ethyl acetate, or glacial acetic acid has also been reported (Hostettmann and Marston, 1995). In the ethanol concentration range of 30–100%, solubility of soyasaponin Bb (soyasaponin I) was maximum in 60% ethanol (Shimoyamada et al., 1993). Solubility of gypsophia saponin in water increased with temperature from 7.4 g/100 mL at 30°C to 18.0 g/100 mL at 70°C (Biran and Baykut, 1975). A sharp increase was observed in the solubility of soyasaponin Bb, which was very low in the acidic region, in the pH range 6.5–7.3 (Shimoyamada et al., 1993). The degree of partitioning of components of crude 70% ethanol extract of soybeans between water and butanol was dependent on the concentration of the extract and pH of the aqueous phase (Shimoyamada et al., 1995). The highest recovery of soyasaponin I in the butanol layer was obtained using 0.04 g/mL of crude extract in the acidic region (about pH 4) (Shimoyamada et al., 1995).

While bitterness is the most common sensory attribute associated with saponins (Price et al., 1985), the occurrence of sweet saponins is also well known (Kennelly et al., 1996). For example, the sweetness of licorice is attributed to its main saponin, glycyrrhizic acid (Figure 1), which is 50 times sweeter than sugar (Muller and Morris, 1966).

The complex structure of saponins may undergo chemical transformations during storage or processing which in turn may modify their properties/activity. The glycosidic bond (between the sugar chain and the aglycone), and the interglycosidic bonds between the sugar residues can undergo hydrolysis in the presence of acids/alkali, due to hydrothermolysis (heating in presence of water) or enzymatic/microbial activity resulting in the formation of aglycones, prosapogenins, sugar residues or monosaccharides depending on the hydrolysis method and conditions (Hostettmann and Marston, 1995). Complete acid hydrolysis yields the constituent aglycone and monosaccharides, whereas under basic hydrolysis conditions, cleavage of O-acylglycosidic sugar chains results in the formation of prosapogenins (Hostettmann and Marston, 1995). The solubility behavior of the parent aglycone can be markedly different than the saponin due to its lipophilic nature (Table 3). DDMP (2,3-dihydro-2,5-dihydroxy-6-methyl-4*H*-pyran-4-one) conjugated saponins, which were determined to be the genuine saponins in intact soybeans, are hydrolyzed into Group B and E saponins upon heating, in alkaline solutions, and in the presence of iron (Kudou et al., 1993; Okubo and Yoshiki, 1995). Soyasaponin βg, which was stable in acidic solution and at temperatures $< 90°C$, was converted into soyasaponin Bb at basic pH and upon heating at 90–100°C (Okubo and Yoshiki, 1995). In the presence of $FeCl_3$, it was degraded into soyasaponin Be and Bb in a ratio of 3:2 (Okubo and Yoshiki, 1995). Deacylation of quillaja saponins was observed upon storage in aqueous solution at pH > 6 (Okubo and Yoshiki, 1995).

The interaction of sterols (Gestetner et al., 1971, 1972; Walter et al., 1954; Shany et al., 1970), minerals (West et al., 1978), and proteins (Potter et al., 1993; Tanaka et al., 1995) with saponins may result in the modification of the physicochemical properties and biological activity of these compounds. Steroid saponins (such as digitonin (Gestetner et al., 1972), alfalfa saponins (Walter et al., 1954)), and triterpenoid saponins (such as lucerne (Gestetner et al., 1971, 1972; Shany et al., 1970)) form water-insoluble addition products with cholesterol and phytosterols such as sitosterol and stigmasterol. Interaction of sterols and lucerne saponins was dependent on the structure of the saponin and sterols (Gestetner et al., 1971, 1972). While cholesterol and β-sitosterol formed complexes with lucerne saponins containing medicagenic acid, which possess carboxyl groups at C23 and C28 positions, saponins with soyasapogenol aglycones did not precipitate (Gestetner et al., 1971). Insoluble complexes were also formed between ammoniated glycyrrhizic acid and alfalfa root saponins and the minerals zinc and iron (West et al., 1978).

The nature and effect of the saponin-protein interaction were dependent on the type of protein (Potter et al., 1993) and the type of the saponin mixture (Tanaka et al., 1995). Upon heating at 78°C (upto 26 min) quillaja saponin interacted with casein to form high molecular weight complexes, whereas soybean proteins formed insoluble aggregates independent of saponin addition (Potter et al., 1993). Similarly, while heating salt soluble proteins from walleye pollack meat at 40–100°C for upto 10 min in the presence of quillaja saponins increased protein aggregation, tea seed saponins inhibited the aggregation of the protein (Tanaka et al., 1995). Complex formation between beet saponin and protein (as evidenced by turbidity and interfacial tension measurements) and destabilization of a model dispersion of sucrose, oil, saponin, and protein in acidic conditions point to the role of beet saponin and protein in the formation of acid beverage floc in sucrose-sweetened carbonated soft drinks and acidified syrups (Morton and Murray, 2001).

The interaction of saponins and proteins also resulted in modifications of protein properties such as heat and enzyme stability (Ikedo et al., 1996; Shimoyamada et al., 1998), and surface properties (Chauhan et al., 1999). Heat stability of bovine serum albumin (BSA) (Ikedo et al., 1996), and resistance of BSA (Ikedo et al., 1996) and soybean protein (Shimoyamada et al., 1998) to chymotryptic hydrolyses improved upon addition of soybean

saponins. The stability of whey proteins to chymotryptic hydrolyses however decreased upon addition of soybean saponins (Shimoyamada et al., 2000). Similarly, unlike soybean protein whose sensitivity to tryptic hydrolysis improved, whey proteins showed higher sensitivity in the presence of soya saponins (Shimoyamada et al., 2000). The influence of soybean saponin on the trypsin hydrolysis of bovine milk α-lactalbumin was attributed to the modification of the protein's tertiary structure (Shimoyamada et al., 2005). Desaponization of quinoa protein increased water hydration capacity and lowered the fat binding and buffer capacity, and total nitrogen solubility (Chauhan et al., 1999). Removal of saponins reduced the emulsion and foaming capacity of the proteins but increased the stability of the foams and emulsions (Chauhan et al., 1999).

Biological Activity

Saponins have been reported to possess a wide range of biological activities, which are summarized and listed alphabetically in Table 4 (Hostettmann and Marston, 1995; Lacaille-Dubois and Wagner, 1996; Milgate and Roberts, 1995; Francis et al., 2002). While crude isolates, extracts, and saponin-containing plants have been utilized in the investigation of biological activity, especially in the earlier studies, developments in the isolation/purification and characterization techniques have enabled the investigation of the bioactivity of well characterized saponins and led to the emergence of structure and bioactivity relationships (Oda et al., 2000; Gurfinkel and Rao, 2003).

The ability of saponins to swell and rupture erythrocytes causing a release of haemoglobin (the *in vitro* haemolytic activity) has been one of the most investigated properties of saponins (Oda et al., 2000). However, even for this activity, which has been related to the saponin structure (type of aglycone and the presence of sugar side chains), there is no apparent consistency between members of this diverse group (Oda et al., 2000).

The toxicity of saponins to insects (insecticidal activity), parasite worms (anthelmintic activity), molluscs (molluscicidal), and fish (piscidal activity) and their antifungal, antiviral, and antibacterial activity are well documented (Lacaille-Dubois and Wagner, 1996; Milgate and Roberts, 1995; Francis et al., 2002). Toxicity of saponins to warm blooded animals is dependent on the method of administration, source, composition, and concentration of the saponin mixture (George, 1965; Oakenfull and Sidhu, 1990). While they show toxicity when given intravenously, their toxicity is much lower when administered orally which has been attributed to their low absorption and the much reduced haemolytic activity in the presence of plasma constituents (Fenwick et al., 1991; George, 1965; Oakenfull and Sidhu, 1990). The results of *in vivo* studies with rats (Yoshikoshi et al., 1995; Gestetner et al., 1968), mice (Gestetner et al., 1968), and rabbits (Gestetner et al., 1968) suggested that saponins are not absorbed in the alimentary channel but hydrolyzed to sapogenins by enzymatic action. A study on the bioavailability of soyasaponins in humans showed that ingested soyasaponins had low absorbability in human intestinal cells and seem to be metabolized to soyasapogenol B by human intestinal microorganisms *in vivo* and excreted in the feces (Hu et al., 2004).

Table 4 Reported biological activities of saponins (Hostettmann and Marston, 1995; Lacaille-Dubois and Wagner, 1996; Milgate and Roberts, 1995; Francis et al., 2002)

Biological Activity
Adaptogenic
Adjuvant
Analgesic activity
Antiallergic
Antiedematous
Antiexudative
Antifeedant
Antifungal
Antigenotoxic
Antihepatotoxic inhibitory effect on ethanol absorption
Anti-inflammatory
Antimicrobial
Antimutagenic
Antiobesity
Antioxidant
Antiparasitic
Antiphlogistic
Antiprotozoal
Antipsoriatic
Antipyretic
Antispasmodic
Antithrombotic (effect on blood coagulability)
Antitussive (relieving or preventing cough)
Antiulcer
Antiviral
Chemopreventive
Cytotoxic
Diuretic
Effect on absorption of minerals and vitamins
Effect on animal growth (growth impairment), reproduction
Effect on cognitive behavior
Effect on ethanol induced amnesia
Effect on morphine/nicotine induced hyperactivity
Effects on ruminal fermentation
Expectorant
Haemolytic
Hepaprotective
Hypocholesterolemic
Hypoglemic
Immunostimulatory effects
Increase permeability of intestinal mucosa cells
Inhibit active nutrient transport
Molluscicidal
Neuroprotective
Reduction in fat absorption
Reduction in ruminal ammonia concentrations
Reductions in stillbirths in swine
Ruminant bloat
Sedative

The safety of saponins of commonly used food and feedstuffs such as soybeans (Ishaaya et al., 1969), and alfalfa (Malinow et al., 1981) has been established by animal toxicology studies. The safety of saponins (such as glycyrrhizic acid) or saponin-containing extracts (such as quillaja extracts) that are used as

Table 5 Lethality of quillaja saponins to CD-1 mice (Kensil and Marciani, 1991)

Dose (μg)	Quil-A	QS-7	QS-18	QS-21
125	1/5	0/5	4/5	0/5
250	2/5	0/5	5/5	0/5
500	4/5	0/5	5/5	1/5

Results are expressed as number of deaths per group of five mice within 72 h after intradermal injection of saponins.

food additives has been the subject of thorough reviews (Joint FAO/WHO Expert Committee on Food Additives, 2004, 2005a; Eastwood et al., 2005). Toxicological recommendations for glycyrrhizic acid are based on its effect of increasing mineralocorticoid activity, which in turn results in electrolyte imbalance due to sodium retention and potassium excretion, and water retention. This effect though reversible can lead to elevated blood pressure if sustained (Joint FAO/WHO Expert Committee on Food Additives, 2005a). Safety evaluation of quillaja extracts takes into consideration the chemical composition of the extracts (such as saponin content, qualitative, and quantitative information on non-saponin constituents) (Joint FAO/WHO Expert Committee on Food Additives, 2004). Quillaja extracts are classified as type 1 and type 2 based on their saponin content, 20–26% and 75–90% respectively (Joint FAO/WHO Expert Committee on Food Additives, 2004), and Acceptable Daily Intake (ADI) values are based on the saponin content of the extracts (Joint FAO/WHO Expert Committee on Food Additives, 2005b). Purification of a saponin extract may result in production of highly potent saponin fractions with varying degrees of toxicities as observed for quillaja saponins (QS-7, QS-18, and QS-21) produced by the purification of an aqueous quillaja extract (Quil-A) (Table 5) (Kensil and Marciani, 1991).

Saponins can impact the immune system through their adjuvant activity, their ability to improve effectiveness of orally administered vaccines by facilitating the absorption of large molecules, and their immunostimulatory effects (Cheeke, 1999). The ability of saponins to act as immunological adjuvants by enhancing the immune response to antigens has been recognized since 1940s (Bomford et al., 1992; Francis et al., 2002). In addition to quillaja saponins, which have been almost exclusively used in the production of saponin adjuvants (Bomford et al., 1992), adjuvant activity of soyasaponins, lablabosides, jujubosides, quinoa, gypsophila, and saponaria saponins has also been reported (Bomford et al., 1992; Oda et al., 2000; Estrada et al., 1998).

Cholesterol-lowering activity of saponins, which has been demonstrated in animal (Oakenfull and Sidhu, 1990; Matsuura, 2001) and human trials (Oakenfull and Sidhu, 1990; Kim et al., 2003b; Bingham et al., 1978), has been attributed to inhibition of the absorption of cholesterol from the small intestine, or the reabsorption of bile acids (Oakenfull and Sidhu, 1990). Feeding animals (poultry, rats, monkeys) diets containing purified saponins or concentrated extracts containing saponins such as digitonin (a steroid saponin obtained from *Digitalis purpurea*), saikosaponin (triterpenoid saponins obtained from roots of *Bupleurum falcatum L.* and related plants), saponaria, soya, chick pea, yucca, alfalfa, fenugreek, quillaja, gypsohila, and garlic saponins resulted in reductions in the plasma and in some cases liver cholesterol concentrations (Oakenfull and Sidhu, 1990; Matsuura, 2001). Recent research highlighted the role of saponins in addition to isoflavones on the hypocholesterolemic effect of soy protein (Lucas et al., 2001; Oakenfull, 2001). The cholesterol lowering effect of dietary saponins in humans is also supported by ecological studies (Chapman et al., 1997). The low incidence of heart disease in the Batemi and Maasai populations of East Africa despite a saturated fat/cholesterol diet, has in part been attributed to the use of plant dietary additives containing saponins in addition to polyphenols, phytosteroids and water-soluble dietary fibre (Chapman et al., 1997).

Anticancer activity has been reported for a number of triterpene and steroid saponins including but not limited to soyasaponins (Rao and Sung, 1995; Kerwin, 2004; Berhow et al., 2000; Plewa et al., 1998), ginsenosides (Huang and Jia, 2005; Liu et al., 2000), saikosaponin-d (Hsu et al., 2004), diosgenin (Raju et al., 2004), and glycyrrhizic acid (Hsiang et al., 2002). Although the potential of soybean saponins as anticarcinogens has been studied in recent years, animal studies are rather limited and most of the evidence comes from cell culture studies (Kerwin, 2004). Methyl protoneogracillin (Hu and Yao, 2003), methyl protogracillin (Hu and Yao, 2001) (steroidal saponins isolated from the rhizomes of *Dioscorea collettii*), protoneodioscin (Hu and Yao, 2002a), and protodioscin (Hu and Yao, 2002b) (furastanol saponins isolated from the rhizomes *of Dioscorea collettii*) have been identified as potential anticancer agents by the National Cancer Institute's (NCI) anticancer drug screen program. Anticancer activities of saponin containing plants such as ginseng and licorice are also being investigated (Wang and Nixon, 2001; Yun and Choi, 1998; Shin et al., 2000). While the cancer preventive effects of ginseng have been demonstrated in experimental models and in epidemiological studies, the evidence on its effect on humans is not conclusive (Shin et al., 2000).

The aglycones, which might be naturally present in the plants or formed by hydrolysis of saponins *in vivo* or during storage and/or processing of the plant material, may have biological activity which is absent or present in a lower degree in their corresponding saponins. The study of the relationship between chemical structure and colon anticancer activity of soybean saponins (as indicated by their ability to suppress the growth of a colon cancer cell line) revealed that the soyasapogenols were more bioactive than the glycosidic saponins (Gurfinkel and Rao, 2003). Other aglycones with anticancer activity include dammarane sapogenins from ginseng (Huang and Qi, 2005), betulinic acid (Yogeeswari and Sriram, 2005; Wick et al., 1999), and oleanolic acid (Liu, 1995; Hsu et al., 1997). Oleanolic acid, one of the most common triterpene saponin aglycone, has also been reported to possess anti-viral (anti-HIV), anti-inflammatory, hepatoprotective, anti-ulcer, antibacterial,

hypoglycaemic, anti-fertility, and anticariogenic activity (Liu, 1995). Anti-viral (anti-HIV), anticancer, antibacterial, antimalarial, anti-inflammatory, anthelmintic, and antioxidant properties have been demonstrated for betulinic acid and its derivatives (Yogeeswari and Sriram, 2005). The conversion of saponins to their aglycones may also result in the loss of activity. For example the hydrolysis of saponins by ruminal bacteria results in the loss of antiprotozoal activity, which requires the intact saponin structure (Cheeke, 1999). Similarly, the deacylation of quillaja saponins decreased their adjuvant activity (Marciani et al., 2002).

COMMERCIAL APPLICATIONS

The diverse physicochemical and biological properties of saponins have been successfully exploited in a number of commercial applications in food, cosmetics, agricultural and pharmaceutical sectors. Market trends towards the use of natural ingredients, and increasing evidence of their biological activity have increased the demand for saponins in recent years (Brown, 1998; Malcolm, 1995). As natural non-ionic surfactants, they find widespread use as emulsification and foaming agents, and detergents (San Martin and Briones, 1999; Balandrin, 1996). Other investigated/proposed applications of saponins and saponin containing plants include as feed additives (Cheeke, 1999; Zhan, 1999; Aoun et al., 2003; Jensen and Elgaard, 2001), as bacterial (Henderson, 2001) and vegetable growth regulators (Yamauchi et al., 2000), and for soil remediation (Roy et al., 1997). While the two major commercial sources of saponins are *Quillaja saponaria* and *Yucca schidigera* extracts (San Martin and Briones, 1999; Balandrin, 1996), a number of other plant materials such as horse chestnut (Indena, 2005), tea seed (Zhan, 1999), and soybeans (Organic Technologies, 2005) are being utilized/evaluated for use as commercial sources of saponins. Pharmaceutical applications of saponins include as raw materials for production of hormones (Blunden et al., 1975), immunological adjuvants (Kensil et al., 2004), and as drugs (Panagin Pharmaceuticals Inc., 2005; Panacos, 2005). Saponins have also been reported to be the active ingredients in various natural health products, such as herbal extracts (Balandrin, 1996).

Food Applications

Yucca (Mohave yucca, *Yucca schidigera* Roezl Fla) and quillaja (quillaia, soap bark, *Quillaja saponaria* Mol Fla) are classified as food additives in the US under section 172.50 (Natural Flavoring Substances and Natural Substances Used in Conjuction with Flavors) (US Food and Drug Administration, 2003). The food additives from natural origins containing saponins used in Japan include enzymatically modified soybean saponin, *Pfaffia paniculata* extract, quillaja extract, tea seed saponins, and yucca foam extract (Japanese Ministry of Health and Welfare, 2005). Quillaja extract is classified by the European Union as a foaming agent for use in water-based, flavored non-alcoholic drinks (E 999; 200 mg/liter calculated as anhydrous extract) (Office for Official Publications of the European Communities, 1996).

Although quillaja and yucca are not considered Generally Recognized As Safe (GRAS) by the US Food and Drug Administration (FDA), they have been given GRAS designation by Flavor and Extract Manufacturers' Association (FEMA) (FEMA #2973, and 3120 respectively) (Ash and Ash, 2002). There is a pending GRAS notice (GRN #165) received by FDA in 2005 from the American Beverage Association for quillaja extract (type 2) to be used as a foaming agent in semi-frozen carbonated and non-carbonated beverages at levels not exceeding 500 milligrams dry weight per kilogram beverage (US Food and Drug Administration, 2005a).

Quillaja extract (type 1) is used in foods and beverages mainly for its foaming properties at concentrations of 100 ppm (dry basis, undiluted extract) in soft drinks, and at concentrations up to 250 ppm in frozen carbonated beverages (Joint FAO/WHO Expert Committee on Food Additives, 2004). Quillaja extract, type 2 is used in Japan as an emulsifier for preparations containing lipophilic colors or flavors that are added to soft drinks, fermented vegetables, and dressing (at claimed concentrations <10 ppm) (Joint FAO/WHO Expert Committee on Food Additives, 2004). Licorice and licorice derivatives, which are considered as GRAS by FDA, are used in foods such as baked foods, beverages, chewing gum, candy, herbs and seasonings, plant protein products, and vitamin and mineral dietary supplements as a flavoring agent only with specific limitations (U.S. Food and Drug Administration, 2005b). Saponins have also been proposed for use in foods as antimicrobial (Sogabe et al., 2003) and anti-yeast agents (Ashida and Matsuda, 1999). Other commercial saponin products for food applications include soybean concentrates marketed as functional food ingredients and nutraceuticals (Organic Technologies, 2005), and a Korean ginseng extract called saponia (Godwithus Co Ltd., 2005).

The presumed health benefits of oleanolic acid led to the development of methods to fortify food products (such as olive oil) with oleanolic acid (van Putte, 2002). Proposed applications for oleanolic acid include as a flavoring agent to modify the aftertaste/taste of the artificial sweetener (Kang et al., 1999) and in fat blends as crystal modifier (Bhaggan et al., 2001).

The physicohemical properties of saponins can also be utilized in food processing applications. Thus, while complex formation of saponins with cholesterol has been used for the removal of cholesterol from dairy products such as butter oil (Micich et al., 1992; Richardson and Jimenez-Flores, 1994), the interaction of saponins with cell membranes has been considered for the selective precipitation of fat globule membranes from cheese whey (Hwang and Damodaran, 1994). In this last application, saponins are used to increase the hydrophobicity of the fat membrane to facilitate flocculation and precipitation of the formed complexes (Hwang and Damodaran, 1994).

Cosmetics

Due to their surface active properties, saponins are being utilized as natural surfactants in cleansing products in the personal care sector such as shower gels, shampoos, foam baths, hair conditioners and lotions, bath/shower detergents, liquid soaps, baby care products, mouth washes, and toothpastes (Indena, 2005; Olmstead, 2002; Brand and Brand, 2004). Natural surfactants containing saponins available commercially include Juazarine from the bark of *Zizyphus joazeiro* tree (Anonymous, 2004), horse chestnut saponins (Indena, 2005) and mixture of plant saponins (Bio-Saponins, Bio-Botanica, Inc., 2005). Saponins and sapogenins are also marketed as bioactive ingredients in cosmetic formulations with claims to delay the aging process of the skin (Yoo et al., 2003; Bonte et al., 1998), and prevent acne (Bombardelli et al., 2001).

Pharmaceutical/Health Applications

Steroid saponin-containing plant materials gained commercial significance in 1950s as raw materials for the production of steroid hormones and drugs. The synthesis of progesterone from the sapogenin diosgenin (Figure 1A) obtained from Mexican yam by Marker et al. in 1940s (Marker et al., 1947) was the beginning of a remarkable era in steroid research culminating in the synthesis of the first oral contraceptive in 1951. Diosgenin isolated from *Dioscorea* species and to a lesser extent structurally similar sapogenins such as hecogenin from *Agave* species have been widely used as raw materials by the steroid industry (Blunden et al., 1975).

Saponins have been used as immunological adjuvants in veterinary vaccine formulations due to their immune enhancing properties since 1950s (Dalsgaard, 1974). Their use in human vaccines, however, has been limited by their complexity and toxicity. Purification of the quillaja extract to yield fractions with differing chemical and biological properties enabled the characterization and thus reproducible production of the fractions for optimal adjuvant activity and minimal haemolytic activity and toxicity (Cox et al., 2002; Kensil and Marciani, 1991). Consequently, there have been significant advances in the development of saponins as human vaccine adjuvants in the last decade leading to the development of a new generation of vaccines against cancer and infectious diseases which are at various phases of clinical trials (Kensil et al., 2004). The use of quillaja extracts (even at concentrations commonly used in foods) as oral adjuvants in human clinical tests requires supporting toxicology and general safety data due to their non-GRAS status (Dirk and Webb, 2005).

The wealth of information on the biological activity of saponins and aglycones from a variety of sources is providing leads for the development of drugs. The chemopreventive and chemotherapeutic activities of ginseng dammarane sapogenins have prompted the development of anticancer drugs which are at various stages of development (Panagin Pharmaceuticals Inc., 2005). A new class of HIV drugs called Maturation Inhibitors (PA-457, in Phase 2 clinical trials) are being developed using betulinic acid derivatives (Panacos, 2005).

Pharmaceutical compositions or plant extracts containing saponins have been patented for the prevention and/or treatment of a variety of conditions such as inflammation (Forse and Chavali, 1997; Bombardelli et al., 2001), infection (Forse and Chavali, 1997), alcoholism (Bombardelli and Gabetta, 2001), pre- and post-menopausal symptoms (Bombardelli and Gabetta, 2001), cardiovascular and cerebrovascular diseases such as coronary heart disease and hypertension (Yao et al., 2005; Hidvegi, 1994), prophylaxis and dementia (Ma et al., 2003), ultraviolet damage including cataract, and carcinoma cutaneum (Satoshi et al., 2004), gastritis, gastric ulcer, and duodenal ulcer (Kim et al., 2003a). The use of saponins in pharmaceutical preparations as adjuvants to enhance absorption of pharmacologically active substances or drugs has also been patented (Kensil et al., 1996; Tanaka and Yata, 1985).

Saponin-containing plants such as ginseng, yucca, horse chestnut, sarsaparilla, and licorice have been used in traditional medicine by various cultures for centuries for the prevention/ treatment of various ailments (Liu and Henkel, 2002; Hostettmann and Marston, 1995). Characterization of the medicinal plants and their extracts points to the role of saponins in conjuction with other bioactive components such as polyphenols in the observed health effects (Liu and Henkel, 2002; Alice et al., 1991). Over 85% of the herbs most commonly used in Traditional Chinese Medicine were observed to contain saponins (in addition to polyphenols) in significant detectable amounts, while the herbal products in the eight best known and most commonly used formulae were explicitly rich in these components (Liu and Henkel, 2002). It should be noted that while some of the health benefits associated with these plants have been supported by clinical data or described in pharmacopeias and in traditional systems of medicine, a variety of uses attributed to these medicinal plants have not been substantiated (Table 6).

EXTRACTION AND PURIFICATION OF SAPONINS AND SAPOGENINS

The recognition of the commercial significance of saponins with expanding applications and increasing evidence of their health benefits have prompted research on process development for the production of saponins on a commercial-scale from natural sources. Existing food processing methods, such as soy protein production, are also being re-evaluated to obtain information on the partitioning of saponins between different process streams (Rickert et al., 2004a, 2004b), which is used to recover saponins as separate fractions (Haokui, 2001), to maximize their retention in the final product (Singh, 2004), and to identify potential raw materials for the production of saponins (Rickert et al., 2004a).

Due to the abundance of saponins in nature, a wide range of plant materials can be used as raw materials for commercial production of saponins. A significant commercial opportunity lies

Table 6 Medicinal uses of licorice (*Radix glycyrrhizae*), ginseng (*Radix ginseng*), and horse chest nut (*Semen hippocastani*) (World Health Organization, 1999a, 1999b, 2001)

MEDICINAL USES	Radix glycyrrhizae	Radix ginseng	Semen hippocastani
Supported by clinical data		As a prophylactic and restorative agent for enhancement of mental and physical capacities, in case of weakness, exhaustion, tiredness, and loss of concentration, and during convalescence.	For treatment of symptoms of chronic venous insufficiency, including pain, feeling of heaviness in legs, nocturnal calf-muscle spasms, itching and oedema. For the symptomatic treatment of chronic venous insufficiency, sprains and bruises.
Described in pharmacopeias or in traditional medicines	As a demulcent in the treatment of sore throats, and as an expectorant in the treatment of coughs and bronchial catarrh. In the prophylaxis and treatment of gastric and duodenal ulcers and dyspepsia. As an anti-inflammatory agent in the treatment of allergic reactions, rheumatism and arthiritis, to prevent liver toxicity, and to treat tuberculosis and adrenocorticoid insufficiency.	Treatment of diabetes, impotence, prevention of hepatoxicity, and gastrointestinal disorders such as gastritis and ulcer.	Treatment of coronary heart disease.
Described in folk medicine, not supported by experimental or clinical data	As a laxative, emmenagogue, contraceptive, galactagogue, antiasthmatic drug, and antiviral agent. In the treatment of dental caries, kidney stones, heart disease, consumption, epilepsy, loss of appetite, appendicitis, dizziness, tetanus, diphtheria, snake bite and haemorrhoids.	Treatment of liver disease, coughs, fever, tuberculosis, rheumatism, vomiting of pregnancy, hypothermia, dyspnoea, and nervous disorders.	Treatment of bacillary dysentery and fevers. Also as a haemostat for excessive menstrual or other gynaecological bleeding, and as a tonic.

in the value-added processing of by-products for the concentration of saponins and/or aglycones such as soybean oil extraction residue (Yoshiki et al., 2005), soy molasses (Dobbins, 2002), asparagus waste (Schwarzbach et al., 2004), and sugarbeet pulp (Sasazuka et al., 1995).

The development of an effective processing methodology starts with the identification of process objectives/product specifications, which is in turn determined by end-product use. The spectrum of saponins with commercial applications ranges from crude plant extracts, which are commonly used for their foaming properties, to high purity saponins with health applications such as vaccine adjuvants, production of which requires a sequence of purification steps. In addition to well-established analytical methodologies, new technologies and approaches are also being investigated to overcome processing challenges posed by the complex nature and diversity of this unique class of compounds. While common trends can be identified, process development is carried out for each raw material as the composition of the plant material and the saponin mixture will affect the process considerably.

Extraction of Saponins

The first step in the processing of saponins involves their extraction from the plant matrix. As in any extraction process, the extraction solvent, extraction conditions (such as temperature, time, pH, solvent to feed ratio), and the properties of the feed material (such as composition and particle size) are the main factors that determine process efficiency and the properties of the end product.

If a purified product is desired, the efficiency of the purification steps needs to be considered while optimizing extraction parameters. For example, conditions maximizing the extraction yield can decrease the selectivity and thus, the purity of the saponins, complicating further purification steps (Wanezaki et al., 2005). The finding that malonyl isoflavones could be separated from soybean saponins easier than other soybean isoflavones due to their higher polarity led to the optimization of the extraction of soybean saponins to be based on malonyl isoflavone content of the extract (Wanezaki et al., 2005).

Sample Pretreatment

Pretreatment steps, which are carried out to increase the efficiency of the extraction, include drying, particle size reduction, and defatting (using a lipophilic solvent such as ethyl acetate or hexane). Defatting can also be carried out after the extraction of saponins. Particle size reduction (grinding) is usually carried out to increase the mass transfer efficiency of the extraction. The variable qualitative and quantitative distribution of saponins in plants enables the selection of the plant part to be used as raw material considering efficiency of the process

and/or extract properties. The efficiency of the separation is improved by using part of the plant with the highest saponin concentration. Selection of the raw material can also be used to overcome processing challenges posed by the other components present. For example, the use of quinoa hulls as raw material for saponin extraction eliminated the problems associated with swelling of starch during extraction of whole seeds (Muir et al., 2002).

Extraction Methods

While traditional solvent extraction methods are commonly used for the production of saponin extracts, recent research focuses on technologies that improve the extraction efficiency by reducing extraction time and solvent consumption/waste without compromising sample quality. Microwave (Vongsangnak, 2004; Kwon et al., 2003a,b,c) and ultrasound (Wu et al., 2001) assisted extractions involve disruption of the internal cell structure and release of intracellular product to facilitate mass transfer, which is achieved by rapid and selective heating of the raw material in a solvent which is (partially) transparent to microwave energy (in microwave extractions) (Kwon et al., 2003a,b,c; Vongsangnak, 2004) and the mechanical effects of acoustic cavitation (in ultrasonic extractions) (Wu et al., 2001).

Commercial applications of Microwave-Assisted Processes (MAP™, microwave technologies patented by Environment Canada) are being currently developed for extraction of natural products such as oilseeds (in collaboration with Bunge Canada, formerly CanAmera Foods, and BC Research) (Environment Canada, 2005) and high value, low volume, natural active ingredients for the pharmaceutical and nutraceutical markets (Radient Technologies Inc., 2005). Ultrasonic liquid processing devices are being used at production level in the pharmaceutical, chemical, petrochemical, and paint industry as well as in the bioprocessing and food industries (Hielscher GmbH, 2005).

Lab-scale microwave and ultrasonic extractions were investigated for the extraction of ginsenosides from ginseng (Kwon et al., 2003b; Vongsangnak, 2004), saponins from chickpeas (Kerem et al., 2005) and glycyrrhizic acid from licorice root (Pan et al., 2000). The ginsenoside yield and composition of a 80% methanol (50 mL) extract obtained from ginseng powder (5 g) using MAP™ for 30 s (4×) (at 72.2°C) were comparable to those of a 12 hr conventional reflux extraction carried out under similar conditions (Kwon et al., 2003b). Similarly, a maximum saponin yield of 7.4 mg/100 mg DW could be obtained in 6 min by microwave-assisted extraction of ginseng (100 mg sample: 15 mL water-saturated n-butanol, 50°C) compared to 8 hr for soxhlet extraction (7.7 mg/100 mg DW; 100 mg sample: 80 mL methanol, 70°C), 6 hr for heat reflux extraction (6.7 mg/100 mg DW; 100 mg sample: 15 mL methanol, 70°C), and 2 hr for ultrasonic extraction (7.6 mg/100 mg DW; 100 mg sample: 15 mL water-saturated n-butanol) (Vong sangnak, 2004). Savings in time and solvent consumption compared to traditional methods such as heat reflux, ultrasonic, Soxhlet extractions, and extraction at room temperature were also achieved by microwave assisted extraction of glycyrrhizic acid from licorice root (Pan et al., 2000). Multi-stage counter-current extraction has also been investigated to improve the efficiency of extraction of glycyrrhizic acid from licorice (Wang et al., 2004).

Pressurized liquid extraction (PLE) involves the use of pressurized solvents at high temperatures. The high temperatures made possible by the application of pressure results in improvements in mass transfer properties of the solvent, hence improving extracting efficiency. The change in solvent polarity hence solubility with temperature of the pressurized solvent coupled with enhanced mass transfer properties makes PLE an attractive method for saponin processing; however, the applications up to date have largely been limited to analytical procedures. In their study on the PLE of medicinal plants, Benthin et al. (1999) compared PLE of escin from CH_2Cl_2-defatted horse chestnut using aqueous methanol (65%) at 140 bar and 100°C with traditional extraction procedure and achieved a higher escin concentration in the pressurized liquid extract (3.73%) than in the traditional extract (2.63%). Extraction efficiency of ginsenosides from *Panax ginseng*, American ginseng and health supplement products using PLE (25–30 bar, 20 minute extraction, 20–25 mL solvent used, 140°C) was comparable to Soxhlet extraction (Lee et al., 2002). The ginsenoside yield of aqueous non-ionic surfactants was higher than that of water (at concentrations higher than critical micelle concentration (0.01%)) and methanol at lower temperatures (Choi et al., 2003). Efficiency of extraction of glycyrrhizic acid from licorice using pressurized methanol (Ong, 2002) (at 100°C, 20 min, 20–25 mL solvent) and pressurized water (Ong and Len, 2003) (at 95°C) was comparable to or higher than that obtained with a multiple step ultrasonic extraction using 70% methanol.

Extraction Solvent

Water, lower alcohols (methanol and ethanol), or water: alcohol mixtures have been widely used for extraction of saponins from plant matrices (Kitagawa, 1986; Bombardelli and Gabetta, 2001). Other solvents investigated for extraction of saponins include aqueous (Choi et al., 2003; Fang et al., 2000) and alcoholic surfactant solutions (Choi et al., 2003), and glycerine (Gafner et al., 2004). The addition of ammonia to solvents for glycyrrhizic acid extraction is based on chemical complexation of glycyrrhizic acid with ammonia, which results in an increase in its extraction yield (Pan et al., 2000).

Supercritical CO_2 has been demonstrated to be a viable alternative to organic solvents for the processing of natural materials with advantages such as ease of solvent removal, solvent free products, and an oxygen free environment. However, the application of $SCCO_2$ technology to the processing of polar solutes such as polyphenolic and glycosidic compounds has been limited by the low solvent power of $SCCO_2$ for these solutes, which can be improved by the addition of cosolvents (Hamburger et al., 2004). The use of cosolvents, however, overrides one of the major advantages of $SCCO_2$ processing: solvent-free processing.

Supercritical CO_2 extraction of ginsenosides from ginseng (Wang et al., 2001), saikosaponins from *Bupleurum chinense DC* (Ge et al., 2000) and glycyrrhizic acid from licorice (Chuanjing et al., 2000; Kim et al., 2004) using cosolvents (ethanol (Wang et al., 2001; Ge et al., 2000), methanol (Chuanjing et al., 2000), and aqueous methanol (Chuanjing et al., 2000)) has been reported. Wang et al (Wang et al., 2001) obtained an oil product containing ginsenosides using $SCCO_2$ extraction of ginseng root hair at 308–333 K and 10.4–31.2 MPa with ethanol. The addition of ethanol to CO_2 (6 mol%) increased the SFE yield of ginsenosides in ginseng oil by a factor of 10 while increasing the yield of the oil by a factor of 4 at 333 K and 31.2 MPa. The enrichment of saponins in plant oils offer interesting product formulations, and may warrant further research. Optimum conditions for recovery of glycyrrhizic acid from licorice were 30 MPa and 60°C for 60 min using $SCCO_2$ + 70% methanol (15% by volume) (Kim et al., 2004).

Effect on Extraction Yield. The choice of solvent for a particular application will be based on the effect of solvent on saponin yield and purity, and the composition of the saponin mixture. Differences between yield and composition of extracts arise from the varying selectivities of the solvents towards individual saponins and other feed components.

The saponin recovery obtained by aqueous alcohol extraction (40–80%) of quinoa hulls was higher than that obtained by pure water or alcohol extractions (Muir et al., 2002). Ultrasound-assisted and Soxhlet extraction of ginseng using water-saturated *n*-butanol gave higher ginsenoside yields than pure and 10% methanol (Figure 2) (Wu et al., 2001). DDMP-saponin yield of 80% ethanol extraction of dehulled peas was higher than that of pure methanol extraction, which was very low (Daveby et al., 1998).

The yield of crude extract of *Glinus lotoides* seeds decreased with the methanol content of aqueous methanol, and the highest crude extract yield (16.5%) was observed with pure water. The highest yield of the *n*-butanol fraction (obtained by the partitioning of the crude extract between water and *n*-butanol) and the purified saponins, however, was achieved by 20 and 60% methanol, respectively (Figure 3) (Endale et al., 2004). The highest total extract yield of MAP^{TM} extraction of ginseng was obtained using 45–60% ethanol, whereas the saponin content increased with ethanol concentration reaching a maximum at 60–75% ethanol (Kwon et al., 2003c). In red ginseng extraction (at 80°C, 5×8 hr), solids yield decreased whereas recovery of ginsenosides increased with ethanol concentration (optimum composition with 70% ethanol) (Sung and Yang, 1985).

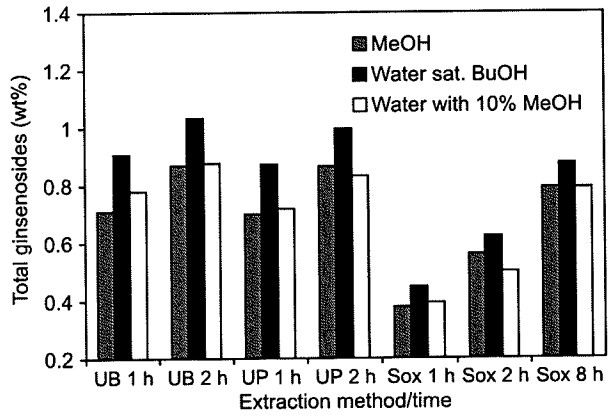


Figure 2 Total ginsenoside yield obtained by extraction of Chinese ginseng root with water, water-saturated butanol, and 10% methanol in UB-ultrasound cleaning bath, UP-ultrasound probe horn, and Sox-Soxhlet extractor (from Wu et al., 2001, Copyright (2001), with permission from Elsevier).

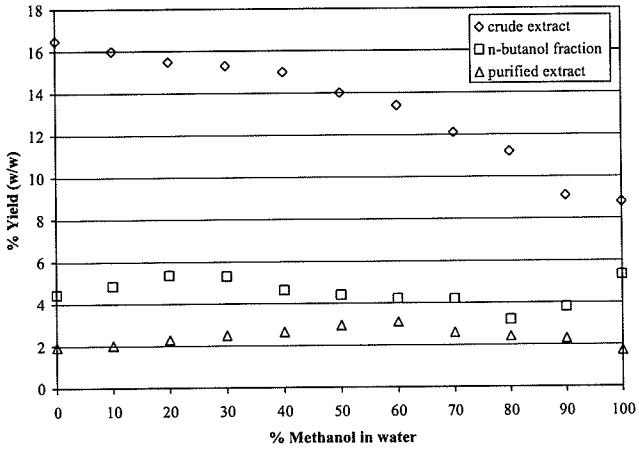


Figure 3 Yield of crude extract, *n*-butanol fraction, and purified extract obtained by extraction of *Glinus lotoides* seeds as a function of solvent composition (Data from Endale et al., 2004).

The recovery of glycyrrhizic acid from licorice using microwave assisted extraction reached a maximum at 50–60% ethanol (Pan et al., 2000). Addition of ammonia to the extraction solvent, which reacts with glycyrrhizic acid to form glycyrrhizic ammoniate, resulted in higher recoveries which were independent of ethanol concentration in 0–60% ethanol range (Pan et al., 2000). No significant difference in glycyrrhizic acid yield was observed between the solvents pure water, 10% ethanol, and 0.5 wt% ammonia in water (Wang et al., 2004).

Effect on Composition of Saponins and Properties of Extracts. The extraction solvent will also affect the composition of the saponin extract. The ratio of neutral to malonyl ginsenosides in aqueous ethanol extract of American ginseng increased with the proportion of ethanol in the solvent (Du et al., 2004). While maximum extraction of neutral ginsenosides was obtained with 70% ethanol, the highest yield of malonyl ginsenosides was achieved using 40% ethanol resulting in the highest total ginsenoside yield with 60% ethanol (Du et al., 2004). Differential extraction of saponins from quinoa bran using pure water and alcohol solvents was reflected in the differences in the saponin composition of the extracts (Muir et al., 2002).

Extraction solvent has also been found to affect the physicochemical properties of the saponin extracts, including particle size, size distribution, morphology, water uptake profiles, sorption isotherms, densities, flow properties, and compaction

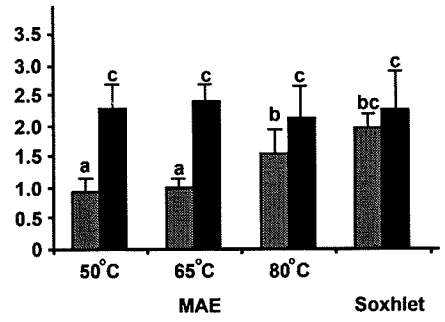


Figure 4 Recovery (g/100 g seed dry weight) of DDMP-saponin obtained by extraction of ground chickpea using microwave assisted extraction (three serial 5-min extractions) and Soxhlet extraction with methanol (black), and 70% ethanol (gray). Bars represent means ± standard deviation ($n = 5$); different letters represent statistical significance level of $p \leq 0.01$ (from Kerem et al., 2005, Copyright Society of Chemical Industry. Reproduced with permission. Permission is granted by John Wiley & Sons Ltd. on behalf of the SCI).

profiles, which are of great significance in pharmaceutical applications (Endale et al., 2004).

Effect of Temperature and Solvent:Feed Ratio on Extraction Efficiency. While temperature was found to have no effect on the microwave-assisted methanol extraction of chickpea saponins, the saponin yield of ethanol:water extracts increased with temperature (Figure 4) (Kerem et al., 2005). Solids (total extract) yield of red ginseng extraction increased while saponin recovery decreased with temperature (particularly at 100°C) (Sung et al., 1985). The multi-stage counter-current extraction yield and glycyrrhizic acid concentration both increased with temperature in the range 30–70°C (Wang et al., 2004). The temperature effect on the composition of aqueous licorice extract was reflected in its flavor characteristics (Vora and Testa, 1997). The low temperature (65.6–82.2°C) extracts had significantly higher glycyrrhizic acid, sugar content, and inorganic salt content, with a mild, sweet flavor, whereas higher temperatures resulted in stronger licorice character with balanced sweetness (Vora and Testa, 1997).

Glycyrrhizic acid concentration in the ethanol extract of licorice decreased with increasing solvent/feed ratio from 339 mg/mL at 6 mL/g to 245 mg/mL at 10 mg/mL while extraction yield stayed in the range of 75–83% (Wang et al., 2004). An increase in recovery of glycyrrhizic acid (%) with microwave assisted extraction was observed with solvent/feed ratio (from 1.88% at 5:1 to 2.58% at 20:1) (Pan et al., 2000). The optimum ratio for quinoa saponin extraction was determined to be 10–15:1 considering extraction yield and practical considerations such as ease of stirring (Muir et al., 2002).

Purification of Saponins

Purification of the crude saponin extract usually requires a sequential approach. A common method for the preliminary purification of saponins after the extraction step involves the partitioning of saponins between aqueous extracts and a water immiscible solvent such as *n*-butanol (Kitagawa, 1986). Further purification can be carried out using solvent precipitation (Kitagawa, 1986; Nozomi et al., 1986), adsorption (Giichi, 1987), ultrafiltration (Muir et al., 2002), and/or chromatography (Kensil and Marciani, 1991). While chromatographic procedures such as open column chromatography, thin layer chromatography, flash chromatography, liquid chromatography (low, medium and high pressure), and countercurrent chromatography have been well established and widely used for analytical scale purification of saponins (Hostettmann and Marston, 1995), their feasibility for commercial scale processing of saponins needs to be evaluated. The purification techniques used in the production of saponins for a variety of applications are discussed below with specific examples.

An aqueous extract of *Quillaja saponaria* bark was separated into 22 fractions (QA1-22) with different adjuvant activity and toxicity using a purification procedure involving methanol extraction followed by silica gel and reverse phase high pressure liquid chromatography (RP-HPLC) (Figure 5) (Kensil and Marciani, 1991).

Due to their high volume of production and increasing evidence on the biological activity of soyasaponins, soybeans (Dobbins, 2002; Giichi, 1987; Bombardelli and Gabetta, 2001), and by-products of soybean processing (Yoshiki et al., 2005) have great potential as raw materials for commercial saponin production. The full realization of this potential in the marketplace however requires development of processing schemes to effectively tackle the associated processing challenges.

The patent "Process for isolating saponins from soybean-derived materials" (Dobbins, 2002) exploits the temperature dependence of solubility behavior of saponins in water:acetone mixtures for the production of a soyasaponin concentrate. An acetone:water (4:1) extraction step (56°C at atmospheric pressure at pH >6.5) followed by cooling the extract led to the precipitation of saponins resulting in a 70% saponin concentrate. Further purification up to 90% was achieved by crystallization.

A soya extract containing 22.5% group B soyasaponin and 15% isoflavones was obtained by reflux extraction with pure or aqueous aliphatic alcohols followed by hexane extraction (for defatting purposes) (Figure 6) (Bombardelli and Gabetta, 2001). In an alternative approach, the defatted soya extract was treated with polyethoxylated castor oil to dissolve the resinous residues and adsorbed onto a polystyrene-based resin. Soya extract containing the isoflavones and saponins were then eluted using 95% ethanol (Figure 6). The soya extract was fractionated into group B saponins and isoflavones using solvent precipitation with aqueous alcohol and a water immiscible protic solvent (such as ethyl acetate) (Figure 6). The fractionation of soya extracts into isoflavone and saponin fractions can also be achieved using an adsorption step (Giichi, 1987; Bombardelli and Gabetta, 2001). The saponin fraction can be further purified using gel filtration and partition chromatography (Giichi, 1987).

Due to the unstable structure of soyasaponin βg, which adds to the complexity and cost of the purification process, group B and E saponins were identified as target compounds in the

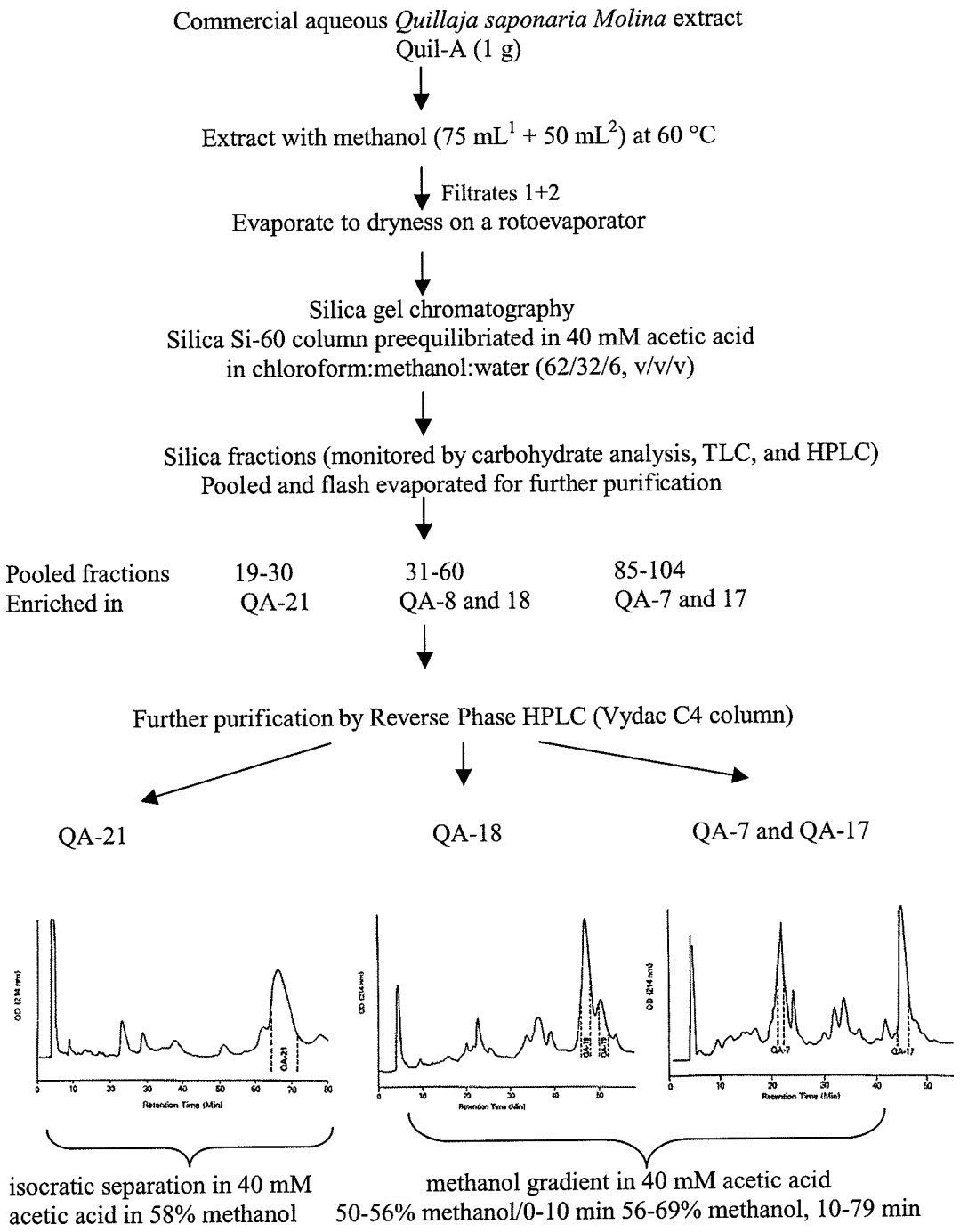


Figure 5 Purification of quillaja saponins for use as adjuvants (adapted from Kensil and Marciani, 1991).

processing of a soybean by-product, the residue of oil extraction, for the isolation of functional soybean saponins (Yoshiki et al., 2005). A fractionation procedure for the production of Group B and E saponin fractions was developed based on information on the chemical characteristics of soyasaponin βg (Figure 7). The soybean glycosides obtained by acidic precipitation were further fractionated into an isoflavone-rich (supernatant) and a DDMP saponin-Fe_2 complex rich fraction (precipitate) by dissolving them in ethanol, mixing with $FeCl_2$ and allowing them to stand overnight. Saponins were further purified by alkaline hydrolysis to remove Fe-DDMP complex, followed by acidic precipitation and partitioning of the precipitate between water and *n*-butanol.

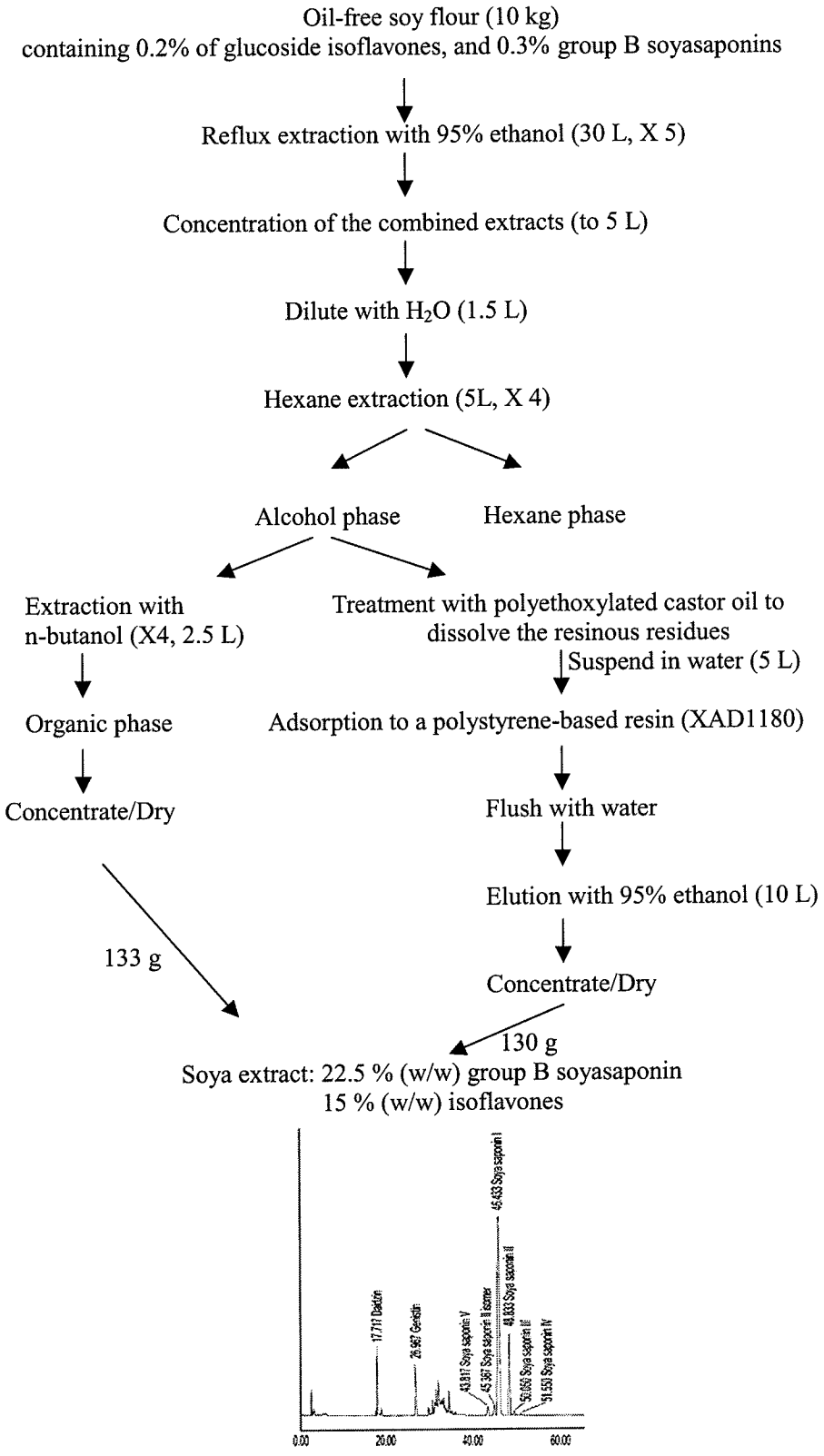


Figure 6 Production and purification of soya extract containing saponins and isoflavones (adapted from Bombardelli and Gabetta, 2001).

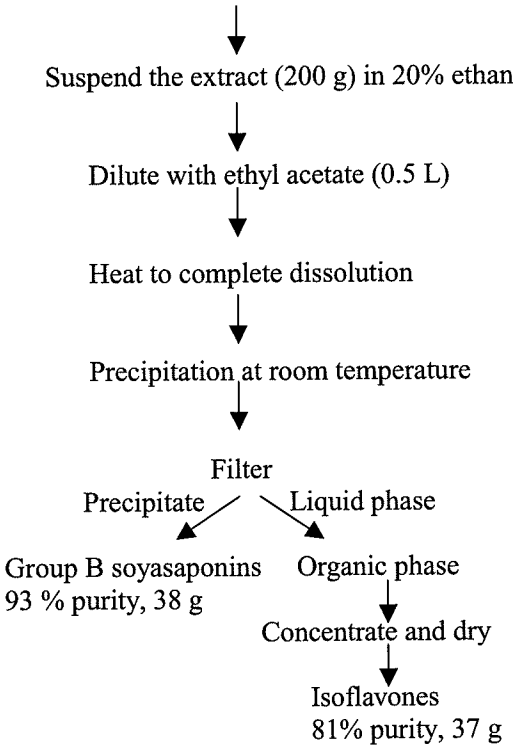


Figure 6 (Continued)

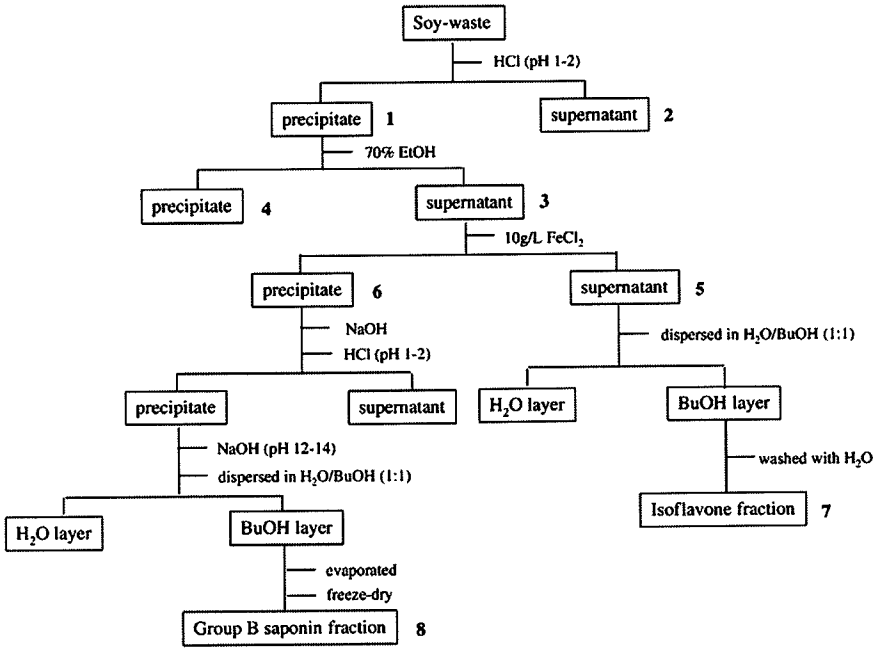


Figure 7 Fractionation of soybean glycosides based on chemical characteristics of soybean saponin βg (from Yoshiki et al., 2005, Copyright (2005), with permission from Elsevier).

The evaporated and freeze-dried *n*-butanol fraction contained Group B (>90%) and E saponins (>10%).

Soyasaponin-I has also been isolated from other legumes including red and white clover, alfalfa, and lucerne using solvent precipitation, adsorption, and heat treatment in an aqueous lower aliphatic solution of an alkali hydroxide (Kitagawa, 1986). One approach involved adsorption of the concentrated extract in water or water:alcohol mixture (≤30%) using a porous, cross-linked polystyrene resin, followed by subsequent elution (with alcohol or alcohol:water mixture), concentration and purification of the saponins by column chromatography on silica gel. Alternatively, the crude saponins were recovered in *n*-butanol by distributional extraction of the concentrated extract. The isolation of soyasaponin-I from the *n*-butanol fraction was then achieved by solvent precipitation (using a soyasaponin soluble and insoluble solvent pair such as methanol and ethyl acetate) followed by treatment with activated charcoal and crystallization from a solvent mixture of chloroform:methanol:water. Alternatively, the *n*-butanol extract was heated in an aqueous lower aliphatic alcohol solution of an alkali hydroxide under reflux, neutralized (by passing it through a column of an ion exchange resin of strong acid type), concentrated, and further purified by column chromatography on silica gel to obtain soyasaponin-I (Kitagawa, 1986).

Quinoa saponin concentrates containing up to 85–90% saponins were produced by ultrafiltration of aqueous alcohol extracts (Muir et al., 2002). Individual saponins were then recovered by Reversed Phase Solid Phase Extraction and preparative RP-HPLC with 98% purity. Solvent (water-*n*-butanol) partitioning, dialysis, and membrane filtration have also been investigated for the recovery of saponins from quinoa (Muir et al., 2002).

A patented process for the isolation of escin from horse chestnut uses the ether extraction of the cholesterol-saponin adduct obtained by treating an aqueous-alcoholic horse chestnut extract with cholesterol and separation of the resulting precipitate (Wagner and Bosse, 1964). Further fractionation of escin into its two isomers of high purity is achieved by converting it into free acid form (by treating it with a cation exchange agent) and heating (50–90°C) until one of the isomers is precipitated due to low solubility in water (Wagner and Bosse, 1963).

Foaming properties of saponins have also been used for the concentration of saponins from unfermented aqueous mixtures (Barbour and Dibb, 1976). A 10–50 fold saponin concentration in the aqueous extract was achieved by foam fractionation with a suitable gas (air, nitrogen and carbon dioxide) (Barbour and Dibb, 1976).

Effect of Processing on Saponin Structure/Properties

As the processing focus shifts from elimination of saponins to their extraction/concentration or retention, information on the effect of processing conditions (such as heat treatment) on the content, structure and properties of saponins becomes a key element in process development. Chemical modification of saponins, as outlined in the section on their physicochemical properties, can take place during processing and/or storage resulting in a change in their total content, composition, and properties/biological activity which may or may not be desirable. Information on the effect of processing conditions on saponins is not only essential to product quality but can also be exploited to customize the saponin properties for a specific application.

Earlier research on the effect of processing conditions on saponins concentrated on the effects of food processing methods such as cooking, soaking, canning, and fermentation on the saponin content of food plants or foods. The decrease in saponin content of foods caused by these processes has been well-documented for a variety of foods such as legumes and quinoa (Anderson and Wolf, 1995; Zhou and Erdman, 1997; Ridout et al., 1991).

The most widely investigated saponin group has been the ginsenosides with a wealth of information available on the effect of various processes such as drying (Du et al., 2004; Popovich et al., 2005), microwave and conventional heating (Ren and Chen, 1999), steaming (Kim et al., 2000), chemical treatment (Kim et al., 1998a), extraction parameters (Du et al., 2004), irradiation (Kwon et al., 1990), and storage (Du et al., 2004) on the concentration of individual ginsenosides and/or their biological activity. The effects of heating, extraction, and storage on oat saponins (Önning et al., 1994), alfalfa saponins (Tava et al., 2003), and soyasaponins (Daveby et al., 1998) have also been documented.

The thermal stability of selected saponins has been investigated by process conditions (time, temperature, pH) and the properties of the saponin. Oat saponins (avenacosides A and B) were heated at 100 and 140°C at different pH to study the degradation during heat processing (Önning et al., 1994). While they were stable up to 100°C for 3 hr at pH 4–7, heating at 140°C especially at pH 4 lead to partial degradation. The degradation rate was significantly increased at pH 4–6 in the presence of catalytic amounts of iron and stainless steel. Drying of American ginseng at temperatures above 40°C resulted in a decrease in the total ginsenoside content (Reynolds, 1998; Du et al., 2004) with a corresponding increase in the ratio of neutral/malonly ginsenosides, which was attributed to the hydrolysis of malonyl to neutral ginsenosides (Figure 8) (Du et al., 2004). The lower thermal stability of malonyl ginsenosides was also documented during heating of American ginseng in 50% ethanol and aqueous extracts (Ren and Chen, 1999). The effect of microwave heating on ginsenoside degradation was the same as conventional heating (Ren and Chen, 1999). A relatively lower thermal stability was also observed for protopanaxadiol than protopanaxatriol saponin (Sung et al., 1985).

Degradation can also occur during extraction and storage as affected by time and temperature (Daveby et al., 1998; Tava et al., 2003). Extraction temperature will be limited by the thermal stability of the target compounds. For example, extraction of glycyrrhizic acid using pressurized methanol was carried out at 100°C as the stability was impaired at temperatures higher than 120°C (Ong, 2002). The malonyl saikosaponins a and d

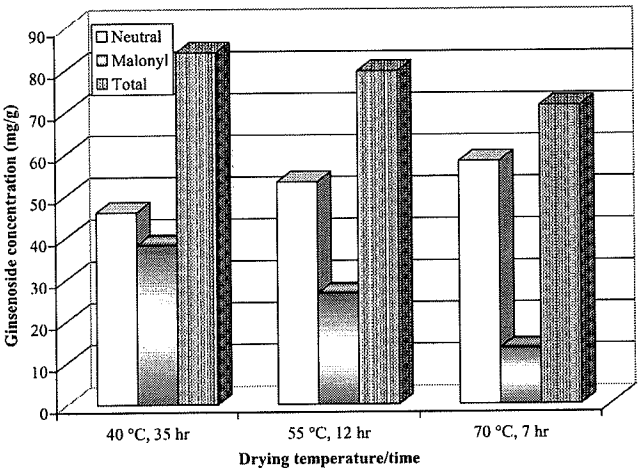


Figure 8 Concentration of ginsenosides in ethanolic extracts obtained from dried ginseng root powder (data from Du et al., 2004).

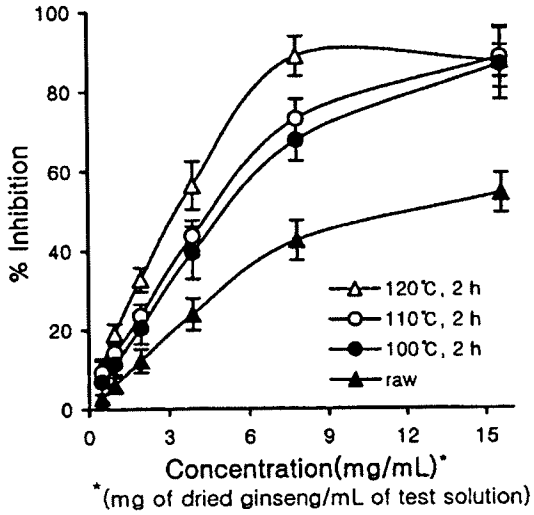


Figure 9 Radical scavenging activity of raw and steamed ginsengs (mean ± sem, $n=5$) (from Kim et al., 2000. Copyright (2000) American Chemical Society, and American Society of Pharmacognosy).

were hydrolyzed by heat and/or acid and the saikosaponins were converted into hydroxylsaikosaponins during the decoction of *Bupleurim falcatum* roots (Ebata et al., 1996). DDMP-conjugated soyasaponin I was converted into soyasaponin I during extraction and storage of dehulled peas (Daveby et al., 1998). Prolonged storage of *Medicago sativa L.* saponins in ethanol resulted in artefact formation due to esterification of acidic saponins with alcohol (Tava et al., 2003). Extraction solvent also affects the properties of the product through its effect on the content and composition of saponins as outlined in the section on extraction solvent.

In the majority of the studies, the effect of processing on saponins has been monitored using the total content or composition of the saponin mixture. Changes in the saponin content/composition, resulting from degradation of saponins present in the raw material and production of new saponins, in turn affect their properties such as bioactivity with significant implications for product quality and product development.

The realization of the enhanced biological activity (antioxidant, anticancer activity) of heat-treated ginseng (such as red ginseng produced by steaming and drying) has put the research focus on the identification of trace compounds formed during heating (Rh_2, Rg_3, Rg_5, Rh_1) and the investigation of their biological activity (Yun et al., 2001; Kim et al., 2000). Steaming of raw ginseng at temperatures >100°C enhanced its biological properties such as its vasodilating (Kim et al., 2000), radical scavenging activity (Figure 9, Kim et al., 2000) and cytotoxicity (Figure 10, Park et al., 2002). The enhanced activity was in turn attributed to the changes in the composition of the ginsenoside mixture induced by the heat treatment (Figure 11). These findings have led to the use of processing as a means to enhance the biological activity of ginsenosides (Park, 2005; Kim et al., 1998b; An et al., 2005). A prodecure containing a series of drying and steaming steps has been used to improve the content of ginsenosides with anticancer activity such as Rg_1, Rg_2, Rg_3, and Rf (An et al., 2005). A ginseng product (sun ginseng) (with a ra-

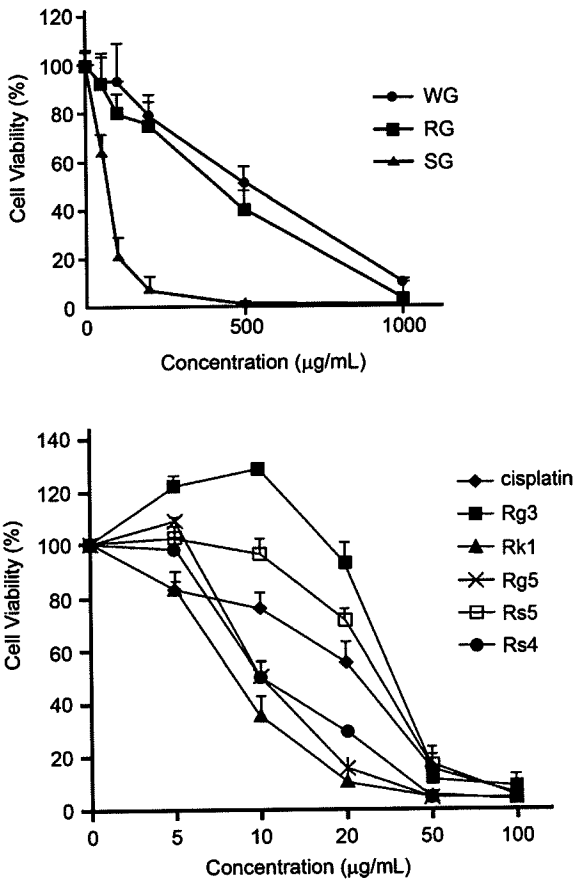


Figure 10 Cytotoxicity of (a) Methanol extracts of white ginseng (WG), red ginseng (RG), processed ginseng (SG, 120°C, 3 h), and (b) Purified ginsenosides (from Park et al., 2002, with permission).

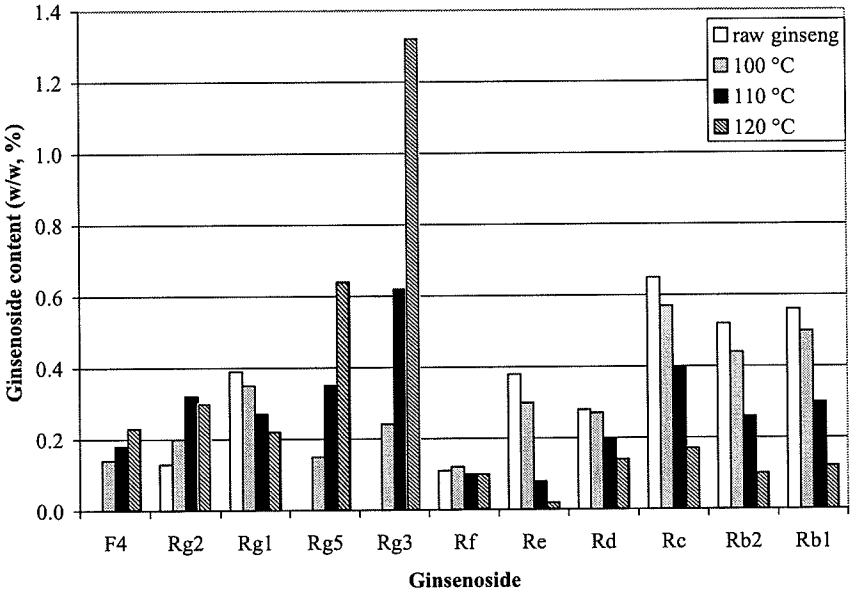


Figure 11 Content (w/w%) of ginsenosides in raw ginseng and ginsengs steamed at 100, 110, and 120°C (data from Kim et al., 2000).

tio of ginsenoside (Rg_3+Rg_5) to (Rc+Rd+Rb_1+Rb_2) above 1) produced by heat treatment at 120–180°C for 0.5–20 hours with enhanced pharmacological effects such as antioxidant and vasodilation activity has also been patented (Kim et al., 1998b).

The hydrolysis of saponins to sapogenins can also modify their bioactivity (as discussed in the section on biological activity). Ginseng sapogenins have been shown to have potent anticancer activity making them the focus of drug development efforts as discussed in the next section.

The biological activity of saponins can also be modified by structural changes induced by activity of enzymes naturally present in the plant material. Enzymatic hydrolysis of bidesmodic saponins retained in the fruit pulp of *Phytolacca dodecandra* berries upon crushing of the berries during aqueous extraction resulted in the formation of monodesmosides with high molluscicidal activity (Ndamba et al., 1994). Similarly, the fungitoxicities of the oat avenacosides were activated by the cleavage of their C-26 bound glucose moiety by α-glucosidase (avenacosidase) contained in oat leaves (Grunweller and Kesselmeier, 1985).

Chemical modification of DDMP-conjugated soyasaponins in soybeans can lead to changes in the quality of soybean foods. For example, while hydrolysis of DDMP saponins can lead to changes in flavor characteristics, the color of the product can be modified by the formation of an insoluble brown complex in the presence of iron (Okubo and Yoshiki, 1994).

Extraction and Purification of Sapogenins

The isolation of sapogenins from plant materials has been widely investigated due to their commercial significance as steroid precursors (Marker et al., 1947; Rothrock et al., 1957). There is renewed interest on production of sapogenins arising from evidence on their biological activities, which are being exploited in a number of applications including pharmaceuticals and cosmetics as described in the section on commercial applications.

Sapogenins can be produced using chemical (Muir et al., 2002; Rothrock et al., 1957), enzymatic (Isaac, 1977), or hydrothermal (Wilkins and Holt, 1958; Wilkins and Holt, 1961) hydrolysis of saponins present in the plant material followed by extraction with organic solvents (such as methanol, ether, ethylene chloride, benzene, carbon tetrachloride, and ethyl acetate) (Rothrock et al., 1957; Hershberg and Gould, 1956; Spensley, 1955) or supercritical fluids (Inada et al., 1990; De Crosta et al., 1993). Alternatively, the hydrolysis can be carried out after solvent extraction of saponins (Wall et al., 1952; Muir et al., 2002) or after expressing the juice containing saponins (Löken, 1975; Miramontes, 1959). Hydrolysis and extraction can take place simultaneously utilizing supercritical fluids (De Crosta et al., 1993; Inada et al., 1990). In their patent on the extraction of plant materials using supercritical fluids, De Crosta et al. (De Crosta et al., 1993) describe a procedure for the extraction of steroid aglycones such as diosgenin and sarsapogenin from plants (barbasco root and Yucca seed, respectively) using CO_2 modified with 10% chloroform and a pressure gradient of 100–300 atm at 250°C, which employs a hydrolysis step during or prior to the supercritical fluid extraction.

Sample pretreatment steps such as incubation with (Miramontes, 1959) or without (Gould and Hershberg, 1956) enzymes (carbohydrases such as cellulase and pectinase) and/or the addition of steroid precursors, saturated hydrocarbons, and plant growth regulators (Hardman, 1971) have been shown to increase

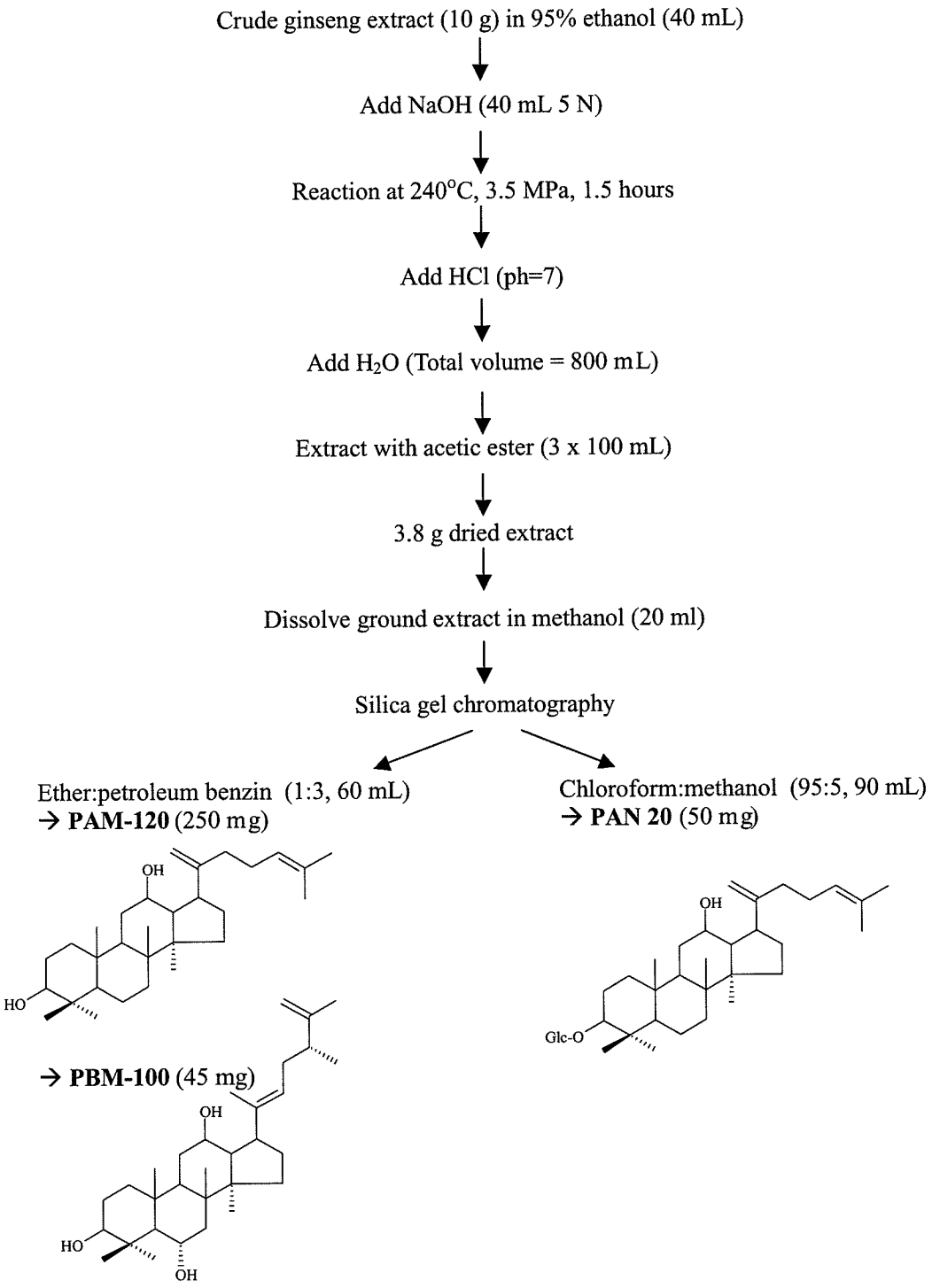


Figure 12 Production of dammarane sapogenins from ginseng (adapted from Huang and Qi, 2005).

the yield of steroid sapogenins. Heating the fermented slurry obtained as waste juice arising from decortication of leaves of the plant *Agave sisalan* to temperatures above 140°C under pressure facilitated the separation of the solids by filtration or centrifugation (Wilkins and Holt, 1958; Wilkins and Holt, 1961).

A recent patent (Huang and Qi, 2005) describes the production of sapogenins from ginseng by reacting a crude ginseng extract with water and a short chain alkali metal alcoholate solution or hydroxide ethanol solution at high temperature (150–300°C) and pressure (2.5–8.4 MPa) (Figure 12). Further purification of the reaction mixture was achieved using silica gel column chromatography to yield novel sapogenins with anti-cancer activity including PAM-120, PBM-110, PBM-100, PAN-20, and PAN-30 (Huang and Qi, 2005).

The aglycones of saponin molecules, such as betulinic acid and oleanolic acid, are also present in nature as isolated molecules. In those cases, their isolation from the plant material only necessitates extraction and purification steps. For example, betulinic acid was extracted from the bark of trees such as *Platanus acerifolia* species using medium polarity solvents such as dichloromethane, chloroform or diethylether followed by crystallization from methanol (Draeger et al., 2001). An herbal extract containing betulinic acid with anticancer activity was produced from ground bark of *Zizyphus jujuba* by macerating the bark in solvent (10–50% aqueous ethanol) (Mukherjee et al., 2004). The recognition of the health benefits of oleanolic acid resulted in the development of processes for the production of extracts containing oleanolic acid from skins of fruits such as apples, pears, cranberries, cherries, and prunes using organic solvents for use in food formulations (Beindorff et al., 2001) and for the fortification of food products such as olive oil with oleanolic acid (van Putte, 2002).

CONCLUSIONS

Saponins include a diverse group of compounds characterized by their structure containing a steroid or triterpenoid aglycone and one or more sugar chains. Their physicochemical and biological properties, few of which are common to all members of this diverse group, are increasingly being exploited in food, cosmetics and pharmaceutical sectors. The full realization of their commercial potential, which is driven by consumer demand for natural products and increasing evidence of their health benefits, requires development of commercially feasible processes that can address processing challenges posed by their complex nature, including their stability. Information on the composition (qualitative and quantitative) and properties of the saponins present in the raw material, and the effects of processing on their composition and properties are key elements of successful process design. The abundance of saponins in nature and their presence in significant quantities in processing by-products (such as by-products of soybean processing) result in a wide range of natural materials that can be exploited for commercial production.

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ANNEXURE 3

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Review

Chromatographic determination of plant saponins

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Abstract

The methods used for saponin determination in plant materials are presented. It is emphasised that the biological and spectrophotomeric methods still being used for saponin determination provide, to some extent, valuable results on saponin concentrations in plant material. However, since they are sensitive to the structural variation of individual saponins they should be standardized with saponin mixtures isolated from the plant species in which the concentration is measured. However, one plant species may contain some saponins which can be determined with a biological test and others which cannot. That is why biological and colorimetric determinations do not provide accurate data and have to be recognized as approximate. Thin-layer chromatography on normal and reversed-phases (TLC, HPTLC, 2D-TLC) provides excellent qualitative information and in combination with on-line coupling of a computer with dual-wavelength flying-spot scanner and two-dimensional analytical software can be used for routine determination of saponins in plant material. The densitometry of saponins has been very sensitive, however, to plate quality, spraying technique and the heating time and therefore appropriate saponin standards have to be run in parallel with the sample. Gas–liquid chromatography has limited application for determination since saponins are quite big molecules and are not volatile compounds. Thus, there are only few applications of GC for determination of intact saponins. The method has been used for determination of TMS, acetyl or methyl derivatives of an aglycones released during saponin hydrolysis. However, structurally different saponins show different rates of hydrolysis and precise optimisation of hydrolysis conditions is essential. Besides, during hydrolysis a number of artefacts can be formed which can influence the final results. High performance liquid chromatography on reversed-phase columns remains the best technique for saponin determination and is the most-widely used method for this group of compounds. However, the lack of chromophores allowing detection in UV, limits the choice of gradient and detection method. The pre-column derivatisation with benzoyl chloride, coumarin or 4-bromophenacyl bromide has been used successfully in some cases allowing UV detection of separation. Standardisation and identification of the peaks in HPLC chromatograms has been based on comparison of the retention times with those observed for authentic standards. But new hyphenated techniques, combining HPLC with mass spectrometry and nuclear magnetic resonance are developing rapidly and allow on-line identification of separated saponins. Capillary electrophoresis has been applied for saponin determination only in a limited number of cases and this method is still being developed.

Keywords: Reviews; Saponins; Triterpene glycosides; Steroidal glycosides

Contents

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1. Introduction

Saponins are widely distributed in plant species, being reported in nearly 100 families. They are naturally occurring glycosides which are found mainly, but not exclusively (lower marine animals) in the plant kingdom. They consist of non-sugar aglycone coupled to sugar chain units. These sugars can be attached as one, two or three sugar chains and the terms monodesmoside, bidesmoside and tridesmoside has been given to these saponins, respectively (Greek *desmos*=chain) [1]. According to the nature of the aglycone they can be classified into steroidal or triterpene groups. Some authors also include within the saponins steroidal glycoalkaloids of solanidans and spirosolan classes. All classes of aglycones may have a number of functional groups (–OH, –COOH, $-CH_3$) causing big natural diversity only because of aglycone structure. Over 100 steroidal and probably even larger numbers of triterpene sapogenins have been identified [2]. This diversity can be further multiplied by the composition of sugar chains, sugar numbers, branching patterns and type of substitution. It is well recognised that even one plant species may possess a number of individual saponins, e.g., alfalfa roots contain at least 25 medicagenic acid, hederagenin, zanhic acid, soyasapogenol and bayogenin glycosides, with the attached number of sugars ranging from one to seven [3]. These structures and their amounts may differ depending on the plant part studied. This structural diversity and resulting wide range of polarities makes determination of individual saponins very difficult.

The early determination of saponins in plant material was based predominantly on gravimetry [4] or on methods taking advantage of some of their chemical or biological features. The foaming property, which is a well-known feature of most saponins, was used to search for plant saponin content. Froth formation after shaking in water solution is specific to most saponins, but some of them, especially those with two or three branched sugar chains do not form stable froth, and conversely some plant extracts not containing saponins may produce froth, providing misleading information.

Some saponins show haemolytic activity which has been used for the development of semiquantitative tests for their determination. In the simplest haemolytic method, saponin-containing material or its water extract is mixed with blood or with washed erythrocytes in isotonic-buffered solution (0.9% NaCl). After 20–24 h samples are centrifuged and haemolysis is indicated by the presence of haemoglobin in the supernatant. For the evaluation of haemolytic activity, European Pharmacopoeia uses as a unit the haemolytic index (HI), which is defined as the number of millilitres of ox blood (2%, v/v) that can be haemolysed by 1 g of crude saponins or plant material. The saponin mixture of *Gypsophila paniculata* L. (HI=30 000) or Saponin white (Merck, HI=15 000) are usually used as reference. Haemolytic indices of saponins are calculated according to the following equation: $HI=HI_{std}\times a/b$; where HI_{std} is the haemolytic index of standard saponin, and a and b are the lowest concentrations of test and standard saponin, respectively, at which full haemolysis occurred. Mackie et al. [5] measured the absorbency at 545 nm of the supernatant after haemolysis and defined one unit of activity as the quantity of haemolytic material that caused 50% haemolysis.

Another modification of the haemolytic method is the haemolytic micromethod [6] in which cow's

blood stabilised with sodium citrate (3.65%, w/v) is mixed with gelatine solution. For this, gelatine (4.5 g) is dissolved in 100 ml of isotonic-buffered solution and 75 ml of this are mixed with 20 ml of blood. The gelatine–blood mixture is spread on a glass plate (10×20 cm) to a thickness of 0.5 mm and, after coagulation, the plates are used for tests. Saponin samples (10 μl) or mashed plant material is placed in localised areas on the gelatine–blood-covered plates and after 20 h the widths of the resulting haemolytic rings are measured. A ring of standard saponin is measured in parallel on each plate. The detailed description of HI and microhaemolytic methods and their limitations in relation to alfalfa (*Medicago sativa*) saponins can be found in a separate publication [7]. It has been clearly documented that saponins differ in their haemolytic activities depending on their structures and also on the haemolytic method used. Monodesmosidic saponins are usually more active than their bidesmosidic analogues [2,7]. The HI method has been used successfully for determination of oleane type saponins in *Aerva lanata* and *A. javanica* [8] and in *Phytolacca dodecandra* [9].

Haemolytic effects can also be used successfully for spotting haemolytic saponins on TLC plates [10,11]. For this, developed plates need to be carefully dried from the solvent residue and then covered with a layer of gelatine–blood solution. After few hours, whitening spots can be seen on plates indicating the presence of saponins. Care has to be taken, as some hydrophobic compounds prevent plates from wetting properly and in fact these areas can be mistakenly identified as haemolytic spots.

Quantitation of saponins can be also performed with other biological methods including growth of sensitive fungus colony *Trichoderma viride* [12] or with *Tribolium castaneum* growth [13] or germination of lettuce seeds [14].

The biological methods, in spite of their simplicity, are at best only approximate and do not distinguish between different saponins. They can be used successfully in work in which the main goal is the comparison of total saponin concentrations, e.g., for breeding purposes where seedlings can be compared quickly without destroying the plants and one mashed leaf is enough for the test. To semiquantify saponins, the test must be standardised with saponin mixtures isolated from the species studied. However, the biological methods fail when saponin preparations are used for biological activity tests. Because the biological activity of saponin has been closely correlated with the chemical structure of individual saponins and because their concentration may change with different factors, including plant age, growth phase, environmental stresses, etc., the exact composition of saponin mixture is crucial. The poor characterisation of saponin preparations is the main cause of difficulties in interpreting findings on their biological activity and differences in the data obtained on the same preparation by different laboratories.

Non-biological methods of total saponin determination include spectrophotometry [15–17], TLC-densitometry, gas chromatography (GC), high-performance liquid chromatography (HPLC), hyphenated techniques such as LC–MS, LC–NMR and capillary electrophoresis (CE). While first three methods were used for quantitation of sapogenins and/or saponins in plant material, recent hyphenated techniques enable rapid initial screening of crude plant extracts, providing preliminary information on the content and the nature of constituents in the matrix. They provide a good method for identification of new compounds with potential biological activity and assure avoidance of unnecessary isolation of common compounds of minor interest [18].

2. Thin-layer chromatographic determination of saponins

2.1. TLC–densitometry

Thin-layer chromatography in one- and two-dimensional (1/2D) modes is a powerful technique which has been used successfully in the separation and determination of a large number of saponins in plant extracts. A major problem with these techniques is first of all the parallel running of the appropriate standards minimising the variation between different plates and colour reactions with spraying agents. The second difficulty is spot detection by means of sophisticated instrumentation for data acquisition and handling to scan the whole plate surface at high speed. This can be achieved, how-

ever, by on-line coupling of a computer with a dual-wavelength flying-spot scanner and two-dimensional analytical software. A great number of saponins are being determined by these techniques and some of them are listed in Table 1.

Most frequently, silica gel plates are used and developing systems consist of choloroform–methanol–water or butanol–acetic acid–water mixtures for saponins and benzene–acetone for aglycones. The most frequently used visualisation sprayers include Carr–Price reagent [30], Liebermann–Burchard reagent [31], phosphotungstic acid [32], 1% $CeSO_4$ in 10 H_2SO_4 [33], 10% H_2SO_4 in EtOH [34], phenol–H_2SO_4 [35], 0.5% *p*-anisaldehyde 1% H_2SO_4 in OHAc [34]. Linear relationships between the peak area and the amount of standard saponins can be found in the range of 1–5 μg per spot with recovery being at the level of 98% and standard deviation of around 3–5%. Comparison of the data with those obtained by HPLC shows that the method is sufficiently accurate for quality control monitoring and is particularly suitable for assays in series [36]. With 2D-TLC it was possible to analyse 35 saponins extracted from stems and leaves of ginseng [37], and *Calendula officinalis* saponins [38]. Fifteen ecotypes of quinoa (*Chenopodium quinoa*) used in breeding programs in the UK were successfully analysed for the concentrations of three groups of saponins, including oleanolic acid, hederagenin and phytolaccagenic acid glycosides [39]. The glycyrrhizic acid in liquorice extracts [40], panaxadiol and panaxatriol [41,42] in ginseng and *Avena sativa* saponins [35] were determined with this technique. The triterpene saponin, escin, in horsechestnut extracts and phytopharmaceutical preparations was measured with high-performance TLC (HP-TLC) with post-chromatographic derivatisation and in situ reflectance measurements. This method enabled determination of large numbers of samples and did not require any tedious clean-up steps prior to analysis, and was highly recommended in pharmaceutical quality control practice.

2.2. TLC–colorimetry

In a large number of investigations, the saponins have been analysed qualitatively/quantitatively with TLC–colorimetry. The saponins can be determined colorimetrically in the crude extract and TLC is just a means of confirmation of their presence in the sample, or the TLC-separated bands are scraped, extracted with alcohol and the extract is treated with a specific reagent. Most frequently used colorants include Ehrlich or vanillin reagents and measurements are made at λ=515–560 nm. The major disadvantage of this procedure is the fact that some other components of the extract such as sterols and bile acids with hydroxyl group at C_3, may give a colour reaction with the reagent, providing misleading information. An anisaldehyde–sulphuric acid–

Table 1
Determination of some saponins by TLC–densitometry

Saponin	Plates	Solvent system	Spray reagent	Refs.
Cucurbitacin B, D,E,I	Silica gel	MeOH–H_2O (55:45)	UV 254 nm	[19]
Cucurbitacin C	Silica gel	EtOAc–C_6H_6 (75:25)	Vaniline–orthophosphate in EtOH	[20]
Ginsenosides	Silica gel G,H	$CHCl_3$–MeOH–H_2O (65:35:10) BuOH–EtOAc–H_2O (4:1:2)	NH_4HSO_4 in in 15% H_2SO_4	[21]
	Silica gel	1,2-Dichloroethanol–BuOH–MeOH–H_2O (30:40:15:25)	NH_4HSO_4 in EtOH	[22]
	Silica gel H	$CHCl_3$–MeOH–H_2O (65:35:10)	10% H_2SO_4	[23]
	Silica gel H	$CHCl_3$–MeOH–H_2O (21:11:4) I dir. BuOH–EtOAc–H_2O (4:1:1) II dir.	Vaniline in H_2SO_4	[24]
Glycyrrhizin	Silica gel 60	BuOH–OHAc–H_2O (5:1:4)	H_2SO_4	[25]
Gypsosides	Silica gel LS	BuOH–OHAc–H_2O (4:1:5)	Phosphotungstic acid	[26]
Oleanolic acid	Silica gel G	$CHCl_3$–Et_2O–MeOH (30:10:1)	Anisaldehyde–H_2SO_4	[27]
	Silica gel G	C_6H_6–Me_2CO (36:13)	10% H_2SO_4	[28]
Triterpene	Silufol	C_6H_6–Me_2CO (8:2)	Phosphotungstic acid	[29]

Table 2
The TLC-colorimetric determination of saponins

Sample	Reagent	Wavelength (nm)	Refs.
Cyclamiretin A	10% vaniline–OHAc, $HClO_4$	560	[45]
Ginsenosides	8% vaniline–EtOH, H_2SO_4	544	[46]
	8% vaniline–EtOH, H_2SO_4	544	[47]
	vaniline–OHAc	560	[48]
	vaniline–$HClO_4$	560	[49]
	HCl–H_2SO4	520	[50]
Protodioscin (I)	Ehrlich	515	[51]
Soyasapogenols	OHAc–H_2SO_4	530	[17]
Steroidal aglycones	Anisaldehydė–H_2SO_4–EtOAc	430	[16]

ethyl acetate reagent gives with steroidal sapogenins a colour reaction which is in general free of any influence from interfering compounds [16]. A good improvement of the colorimetric procedure can be the application of a reversed-phase clean-up stage (SPE) prior to the determination. Depending on the nature of saponins, their retention on reversed-phase support is different and appropriate selection of the solvents allows their considerable purification. This was successfully applied for soybean [43] and alfalfa [44] saponins. This clean-up procedure is a very useful technique also for purification of extracts prior to HPLC determination.

When silica gel TLC is used prior to colorimetric determination, a considerable portion of the compound can be adsorbed irreversibly in the stationary phase and the quantities present may be underestimated. To use this procedure for routine analysis, it first has to be calibrated against a more sophisticated technique, e.g., HPLC. Examples of the TLC–colorimetric determinations are presented in Table 2.

3. Gas–liquid chromatography

A basic requirement for a compound to be analysed by GC is its volatilisation. Saponins as polar and quite large molecules are not easily volatile compounds and they have to be volatilised by derivatisation to acetyl, methyl or trimethylsilyl ethers. This method has been used almost exclusively for the separation of aglycone moiety (Table 3). However, a few attempts have been made to use gas chromatography for the determination of intact saponins and these showed that analysis of trimethylsililated glycosides possessing four or less sugar units was satisfactory [63]. However, these sugars have to be attached not through an ester linkage, otherwise they undergo deglycosidation reaction in the injection port of the gas chromatograph [64].

The first step in GC analysis of sapogenins is hydrolysis of intact saponins to their aglycones. This is one of the crucial points of analysis since hydrolysis products are not always natural genins but depending on the saponin structure, time and conditions of hydrolysis they may form number of artefacts. For instance, hydrolysis of soyasaponins present in soybean, alfalfa and a number of other legumes leads to the formation of several aglycones (Table 4). Their relative quantities change significantly depending on the time of hydrolysis and also on the type of hydrolysing agent. Soyasapogenols A and E seem not to be to sensitive to these parameters, while the concentration of soyasapogenol B decreases with gradual increase in soyasapogenols C, D and F, the artefacts of soyasapogenol B degradation. Also the solvent used in the hydrolysis mixture seems to influence the final results, and creation of artefacts is possible in aqueous hydrolysis. Similar results were obtained by Ireland and Dziedzic [66], where in aqueous hydrolysis soyasapogenol B_1, C, D and E were registered while genuine aglycones soyasapogenol A and B were obtained when soysaponins were hydrolysed with sulphuric or hydrochloric acid in anhydrous methanol [67].

The situation becomes even more complicated

Table 3
The gas–liquid chromatography analysis of saponins

Sample	Saponins	Column	Refs.
Alfalfa	Medicagenic acid, hederagenin soyasap. B,C,D,E,F, oleanolic acid (methylated-acetylated)	OV-1	[52]
	Medicagenic acid dimethyl ester, di(trimethylsilyl)	OV-17 on Chromosorb or 3% SP-2250 on Supelcoport	[53]
	Soyasapogenols A-E, medicagenic acid TMS	OV-17 on Chromosorb W HP	[54]
	Medicagenic acid TMS	SP-2250 on Supelcoport	[55]
Ginseng	Ginsenosides TMS	OV-101 on Chromosorb WHP	[32]
Legumes	Soyasapogenol B TMS	Technochrom SPB-1	[56]
	Soyasapogenol A, B TMS	OV-1 on Diatomit CQ AW DMCS	[57]
Lentil	Soyasapogenol B TMS	DB-1, 0.25 micron film	[58]
Lupin	Soyasaponin I, VI TMS	DB-1, 0.25 micron film	[59]
Quinoa	oleanolic acid TMS	SE-30 on Chromosorb W AW DMCS	[60]
Soybeans	Soyasap. I, II, III, A1, A2 TMS	FFAP on Chromosorb GAW DMCS	[33]
	Soyasapogenol A, B TMS	OV-1 on Chromosorb	[33]
Triterpene	Oleanane, ursane type TMS	OV-101	[61]
	Oleanolic and ursolic acids	OV-17	[62]

when saponins possessing different aglycones are hydrolysed. The complete hydrolysis of all saponins into sapogenins seems to be a key step for their quantitation. Tava et al. [52] showed that the optimal conditions for hydrolysis of alfalfa root saponins was obtained upon refluxing for 8 h with 2 *M* HCl in 50% aqueous methanol. Prolonged times of hydrolysis gave reduced medicagenic acid yields, probably due to decomposition. Under these conditions, soyasapogenol B was almost completely decomposed to yield the artefacts (Fig. 1). Similar changes in medicagenic acid concentration versus time of hydrolysis were reported [55]. The best results were obtained when the reflux time was 12 h and the sulphuric acid concentration was 0.5 *M* in 1,4-dioxane–water (1:3, v/v).

A number of saponins generate, during hydrolysis, not the genuine aglycone but a mixture of it and the lactone. This can happen in case of the glycosides of hederagenin and oleanolic acid [68,69], jujubogenin→ebelin [70], cochalic acid→echinocystic acid [71], zanhic acid→lucernic acid [72], and some others. Acid-catalysed double-bound migration [73], epimerisation [71], and dehydration [74] are often observed during hydrolysis. The same is true with steroidal saponins where, for instance, furostanol saponins in the presence of methanol can be converted to their 22-OCH_3 derivatives. This great number of possibilities for artefact formation makes GC analysis complicated.

Identification of derivatised aglycones can be performed by comparison of retention times of peaks in the sample GC spectrum with the retention times of appropriate standards. Best resolutions with relatively short retention times are obtained for trimethylsilyl derivatives. However, these derivatives are rather difficult to use in GC–MS since identification of the MS peaks is complicated. Rao and Bories

Table 4
Influence of the conditions and the time of hydrolysis of alfalfa saponins on soyasapogenol concentration [65]

Hydrolysing agent, time	Soyasapogenol concentration (% of total)					
	A	B	C	D	E	F
2 N H_2SO_4 in 50% MeOH, 1 h	21.9	55.2	6.9	7.6	1.9	6.3
2 N H_2SO_4 in 50% MeOH, 30 h	23.2	12.5	22.4	21.6	1.7	18.6
1 N H_2SO_4 in dioxan–water (1:3), 8 h	24.5	48.3	11.3	–	2.2	13.7

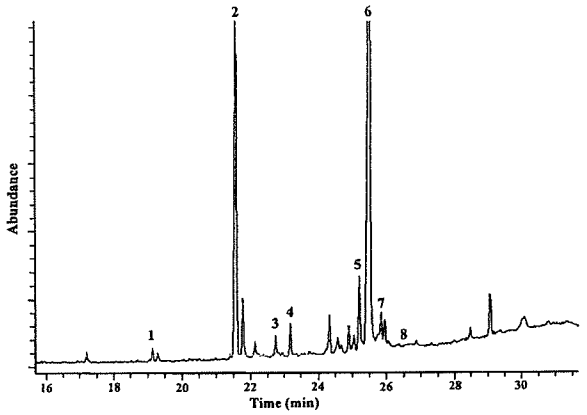


Fig. 1. GC analysis of derivatized sapogenins from alfalfa roots: (1) soyasapogenol C; (2) betulinol (internal standard); (3) hederagenin; (4) soyasapogenol D; (5) soyasapogenol E; (6) medicagenic acid; (7) soyasapogenol F; (9) soyasapogenol B. Column: DB5 (30 m×0.32 mm I.D.; 0.25 μm film thickness). Chromatographic conditions: injector, 310 °C; detector, 340 °C; column temperature, 90–300 °C; carrier gas, He.

[55] compared GC separation of di-*o*-acetyldimethyl ester of medicagenic acid with its trimethylsilyl derivative on an OV-17 column and reported that much higher temperatures and longer times were necessary to achieve the elution of acetylated and/or methylated sapogenins. In spite of these difficulties, a mass spectral study performed on medicagenic acid present in a mixture of acetylated and methylated sapogenins using GC–MS displayed a base peak at *m/e* 203 due to the expected retro-Diels-Alder fragmentation, together with other characteristic ions at *m/e* 262, 494, 554 and 614 (M^+).

4. High-performance liquid chromatography

High-performance liquid chromatography is the most powerful and the most frequently used technique for saponin determination due to the fact that it can deal effectively with non-volatile, highly polar compounds. It has been used extensively for determination of both aglycones and intact saponins. The separations are performed usually on normal (silica gel) and reversed-phase (C_8, C_{18}) columns, of which C_{18} has been definitely preferred, but other modified silica gel supports including NH_2 and DIOL are occasionally used (Table 5). In some instances when resolution of saponins on reversed-phase is insufficient, the selected carbohydrate, borate anion-exchange and hydroxyapatite [$Ca_{10}(PO_4)_6(OH)_2$] supports have been used successfully. Carbohydrate and NH_2-modified columns have been shown to be very effective in the separation of glycoalkaloids [102] but some steroidal saponins can also be successfully analysed [135] The chaconines and solanine were nicely separated with Bondapack NH_2 column in the reversed-phase mode in less than 7 min (detection limit, 5–15 ppm) [100] The borate anion-exchange chromatography depends on the formation of borate complexes with *cis*-diols in the saccharide moiety. The formation of these complexes in some cases improves significantly the resolution and separation of isomeric glycosides which are not separated on reversed-phase support [98]. After separation, genuine saponin can be recovered by removing borate as volatile methyl borate by repeated co-distillation of the eluate with methanol. Resolution of closely related compounds can be also improved by the application of hydroxyapatite support. The hydroxyapatite is more hydrophilic than silica gel support and allows separation of two glycosides differing only in a terminal pentose [99].

The main problem in HPLC analysis of saponins is detection. Since only a few saponins, for instance glycyrrhetinic acid and its glycosides and cucurbitacins, have absorption maxima in UV range, they are the only compounds that can be easily detected at 254 nm. The majority of saponins do not possess chromophores necessary for UV detection, and the separation of intact glycosides or their aglycones has to be traced at lower UV wavelengths ranging from 200 to 210 nm. The sensitivity with this detection mode has been satisfactory and depending on the nature of the saponin ranges from 50 ng for avenacoside B [81] to 300 ng for ginseng saponins [92]. The detection at lower wavelengths, however, limits the selection of solvents and the gradients that can be used. Since acetonitrile gives much lower absorption at lower wavelengths than methanol, the selection of acetonitrile–water gradients is the mode of choice. Similarly, the gradient cannot cover a wide range of concentrations due to the baseline drift, which creates an additional problem with saponin analysis. The bidesmosidic saponins elute in

Table 5
The high-performance liquid chromatographic determination of saponins

Sample	Column	Solvent system	Detection (nm)	Refs.
Aescin	C_{18}	MeCN–H_2O	206	[75]
Aesculus sap.	C_{18}	MeCN–H_2O–H_3PO_4	210	[76]
	C_{18}	MeCN–H_2O–H_3PO_4	205	[77]
Agave sapogen.	C_8	MeCN–H_2O (benzoate esters)	254	[78]
Amaranthus sap.	C_{18}	MeCN–H_2O (Br derivatives)	260	[79]
Aster sap.	C_{18} Nova-Pak	MeCN–H_2O	210, TIC[a]	[80]
Avenacosides	RP8	MeCN–H_2O	200	[81]
Calendula sap.	C_{18}	Me–OH–H_3PO_4	210	[82]
Cucurbitacins	C_{18}	MeOH–H_2O (45:55 or 70:30)	254	[20]
Diosgegnin acet.	Silica gel	Hexane-*iso*-PrOH	219	[83]
Ginsenoside Rb1	Lichrosorb-NH_2	MeCN–H_2O–BuOH (8:2:1)	210	[84]
Ginsenosides	Silica gel LS-310	*n*-C_6H_{14}–CH_2Cl_2–MeCN (15:3:2) Benzoyl	240	[85]
	Silica gel	*n*-Heptan–BuOH–MeCN–H_2O	207	[86]
	Silica gel	CH_3Cl–MeOH–H_2O (30:17:2)	RI[a]	[87]
	Spherical silica gel	Hexane–Et_2O–EtOAc	206	[88]
	Carbohydrate	MeCN–H_2O (80:20–94:6) MeCN–H_2O–BuOH (86:14:10)	210	[89]
	Bondapack/carbohydr.	MeCN–H_2O–BuOH (80:20:15)	RI	[90]
	Bondapack C_{18}	MeCN–H_2O (1:1–9:1)	220	[91]
	C_{18}	MeOH–H_2O	203	[86]
	C_{18}	MeCN–H_2O	203	[92]
	C_{18}	MeOH–H_2O	203	[93]
	C_{18}	MeCN–50 n*M* KH_2PO_4 MeCN–H_2O–H_3PO_4	202	[94]
	C_{18}	MeCN–H2O–H3PO4	205	[95]
	C_{18}	MeCN–H_2O	203	[96]
	C_{18}	MeCN–H_2O	203	[97]
	Anion-exchange	MeCN–0.25 *M* H_3BO_3 (12.5:87.5)	UV	[98]
	Hydroxyapatite	MeCN–H_2O (80:20) MeCN–H_2O (90:10 →70:30)	210	[99]
Glycoalkaloids	Bondapack NH_2	THF–H_2O–MeCN (56:14:30)	208	[100,101]
	NH_2	MeCN–KH_2PO_4–H_2O	208	[102]
	Carbohydrate	MeCN–THF–H_2O	215	[103]
	C_{18}	MeOH–H_2O–H_3PO_4	206	[104]
	C_{18}	MOH–0.01 *M* Tris, MeCN–0.01 *M* Tris	205	[105]
	C_{18}	MeCN–H_2O–ethanolamine (45:55:0.1)	210	[106]
	YMC-Pack SiO2	$CHCl_3$–MeOH–NH_4OH (10:13:1; 7:13:2)	230	[107]
Glycyrrhizin	C_{18}	MeOH–H_2O–TBA–H_3PO_4	254	[108]
	C_{18}	MeOH–H_2O–OHAc	254	[109]
	C_{18}	MeOH–H_2O–$HClO_4$	254	[110]
	C_{18}	MeCN–MeOH–H_2O+ammonium perchlor.	254	[111]
Gypenosides	C_{18}	MeOH-H_2O	210	[112]
Gyspogenin	$glcAC_{18}$	MeOH-H_2O-TBA-H_3PO_4	206	[113]
H. helix sap.	C_{18}	MeCN–H_2O	205	[114]
Medicagenic acid	C_{18} Hypersil	MeOH–H_2O–HCOOH	210	[115]
Medicagenic acid glc	Eurospher C_{18}	H_2O–MeCN (10–90%) Br-derivatives	260	[116,117]
Nerium sap.	C_{18} Nova Pak	MeCN–H_2O–TFA (20:80→45:55)	210, TIC	[80]
Oleanolic acid	C_{18}	MeCN–H_2O	210	[118]
Oleane glc.	Bondapak C_{18}	H_2O–MeCN (70:30→ 50:50)	206	[119]
		H_2O–MeCN (40–70%) Br-derivatives	254	[119]
	RP-8, DIOL	H_2O–MeCN (85:15	206	[120]

Table 5. Continued
The high-performance liquid chromatographic determination of saponins

Sample	Column	Solvent system	Detection (nm)	Refs.
Primula sap.	C_{18}	MeCN–H_2O–H_3PO_4	195	[121]
Ruscin	C_{18}	MeCN–H_2O	202	[122]
Saikosaponin	Aquasil, hydroxyapatite	H_2O–MeCN, $CHCl_3$–MeOH–H_2O	205	[123]
	Develosil-ODS	MeOH–H_2O	210	[124]
	Nucleosil 50-5	$CHCl_3$–MeOH–EtOH–H_2O (62:16:16:6)	210	[87]
	Silica gel	$CHCl_3$–MeOH–H_2O (30:10:1)	RI	[87]
	C_{18}	MeCN–H_2O	210	[124]
	C_{18}	MeOH–H_2O–OHAc-trimethylamine	254	[125]
Soyasapogenols	Silica gel	Petrol–EtOH	MD[a]	[126]
Soyasaponins	Lichrosorb RP18	MeOH–PrOH–H_2O–OHAc (32:4:63:0.1)	RI	[127]
	Silica gel	$CHCl_3$–MeOH–H_2O–OHAc	MD	[128]
	C_{18}	MeCN–H_2O (coumarin derivatives)	Fluor.[a]	[129,130]
	C_{18}	MeCN–*n*-PrOH–H2O–OHAc (32:4:63:0.1)	205	[131]
Soyasaponin VI	Ultrasphere C_{18}	MeCN–OHAc (1000:0.3) (S1) H_2O–OHAc–EDTA (1000:0.3:0.15) (S2)	205	[132–134]
Steroid saponin	Silica gel	Hexane–EtOH–H_2O	208	[135]
	NH_2	MeCN–H_2O	208	[135]
Steroidal sapog.	Silica gel	Hexane–Me_2CO, hexane–EtOH	206	[136,137]
	C_{18}	MeCN–hexane–THF, MeOH–H2O	206	[136,137]
Swartzia sap.	C_{18}	MeCN–H_2O–TFA (30:70→50:50)	210, TIC	[80]
Triterpene sap.	Alltech C_{18}	H_2O–MeCN (20–80%)	210	[138]
	Hydroxyapatite	MeCN–H_2O (87:13; 80:20)	210	[99]
Zanhic acid glc.	Spherisorb C_{18}	H_2O–MeCN (10→90%) Br-derivatives	260	[139]

[a] TIC, total ion current; RI, refractive index detector; MD, mass detector; Fluor., fluorescence detector.

water–acetonitrile gradient at relatively low concentrations of MeCN while monodesmosides elute later and are consequently much more difficult to quantify. Some plant extracts may contain large numbers of glycosides differing in polarities due to the number of sugars attached. Analysis of such a complicated mixture would need wide range of solvent concentration. Application of a gradient completely excludes detection with a refractive index detector (RI) and this is why this type of detection has been rarely used.

The alternative to low wavelength UV or RI detection has been pre-column derivatisation of saponins in order to attach a chromophore that facilitates UV detection at higher wavelength (254 nm). Several attempts has been made to introduce chromophores. Kitagawa et al. [129] employed the derivatisation of saponins from soybean with coumarin and the registration of HPLC profiles with a UV-fluorescent detector. Similar attempts have been made by others to introduce benzoyl chromophore through the pre-column reaction of saponins with benzoyl chloride in pyridine and detection at 254 nm [76,85]. These derivatisation procedures, however, create some practical problems due to the steric shape of the saponin molecule and differentiated rate of substitution of functional –OH groups. Some of these groups are not readily derivatised, and thus the reaction mixture of a single saponin may contain a number of its derivatives. During the derivatisation of complex saponin mixtures many peaks are observed and their interpretation and quantification are difficult.

Promising results has been obtained by derivatisation of saponins with 4-bromophenacyl bromide in the presence of crown ether. Originally this derivatisation mode was employed for the analysis of fatty acids and prostaglandins. Slacanin et al. [119] applied this procedure for the determination of oleane saponins in *Phytolacca dodecandra*. To be derivatised with 4-bromophenacyl bromide, a saponin molecule has to possess at least one carboxyl group, either at the aglycone or sugar part. Therefore, this method can be applied only to some groups of saponins. The oleanolic acid monodesmosides of *P. dodecandra* could be easily derivatised, while

bidesmosides had to be determined in a separate HPLC run with 206 nm detection. This method was excellent for saponin determination in the roots and aerial parts of alfalfa [116,117]. The medicagenic acid glycosides, both in monodesmosidic and bidesmosidic forms can be chromatographed after derivatisation in a single run on a C_{18} column (Fig. 2). Soyasaponin I and monodesmosides of hederagenin can also be determined. Zanhic acid tridesmoside in the aerial part of alfalfa had both –COOH groups glycosylated and could not be readily determined. Due to the very high polarity of this compound, determination at 210 was also very difficult. This problem can be avoided by alkaline hydrolysis of zanhic acid tridesmoside prior to the derivatisation with 4-bromophenacyl bromide. The resulting prosapogenin derivatises readily and can be chromatographed at 260 nm [139]. To apply 4-bromophenacyl bromide derivatisation to the analysis of mixtures of mono-, bi- and tridesmosidic saponins, the method can be modified to allow chromatography of all three groups. For this, two HPLC runs are necessary. In the first run, only monodesmosides or bidesmosides with one free –COOH are determined. For the saponins not having free –COOH group, the plant extract is treated with saturated lead acetate solution in H_2O and the resulting precipitate is centrifuged at 3000 *g*. The precipitate contains all carboxyl-free saponins, and the supernatant is passed through a C_{18} Sep-Pak cartridge which retains saponins. They are then washed with methanol, alkaline hydrolysed and derivatised for chromatography [117].

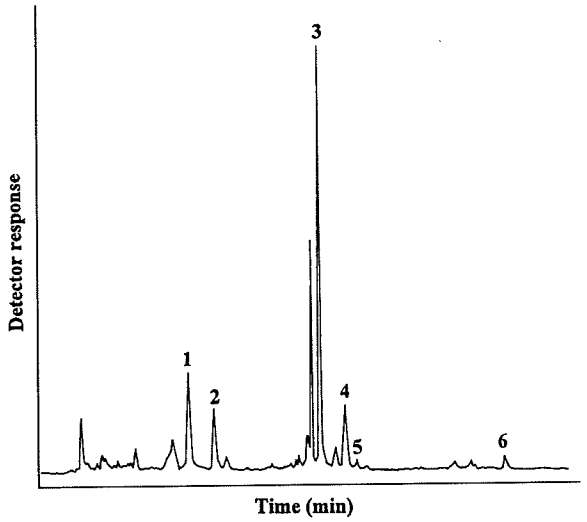


Fig. 2. HPLC separation of 4-bromophenacyl bromide derivatives of saponins from alfalfa roots: (**1**) 3Glc,28AraRhaXyl medicagenic acid; (**2**) 3Glc,28Glc medicagenic acid; (**3**) 3GlcA,28AraRhaXyl medicagenic acid; (**4**) 3AraGlcAra hederagenin; (**5**) soyasaponin I; (**6**) 3Glc medicagenic acid (for structures see Fig. 3). Column, Eurospher C_{18}, 5 μm (4.6×250 mm); eluent, linear gradient 20–80% acetonitrile in water; flow-rate, 1 ml/min; detection, 260 nm.

No matter which method of detection is used for HPLC determination of saponins, a solid-phase clean-up step is highly desirable prior to analysis. The C_{18} [43,44,76,95,96,128,139,140] for saponins and NH_2 [102] Sep-Pak cartridges for glycoalkaloids have been used successfully, with recovery of glycosides higher then 95%. All carbohydrates and most of the phenolic compounds can be removed from the matrix by the careful selection of eluent, usually methanol–water mixture. The carbohydrates pass readily C_{18} Sep-Pak with water, and most of the phenolic acids and flavonoids can be removed with 40% methanol at which concentration the majority of saponins are retained on the solid-phase [44]. The saponins removed with methanol are highly purified and HPLC profiles are much easier to interpret. The solid-phase clean-up procedures by simplifying extraction and purification ensure mild conditions for these processes and protect saponins from multiple artefact formation [141].

5. Hyphenated techniques in HPLC

The HPLC methods for the determination of saponins do not ensure identification of individual peaks because the absorption spectra are not specific. This is not like in the case with the determination of phenolics where absorption spectra registered with a photodiode array detector (DAD) provide useful information about the type of chemical and its oxidation pattern. Identification of saponins must rely exclusively on the comparison of retention times of unknown peaks with retention times of an appropriate saponin standards, for example alfalfa saponins [116]. None of the detection modes permits the registration of all the saponins encountered in plant extracts within a single analysis. To overcome

this problem, some authors have used off-line peak identification with high-performance thin-layer chromatography (HPTLC), multiple internal reflection infrared spectroscopy (MIR-IR) and field desorption mass spectrometry (FD-MS) [92,96,142].

More recently, hyphenated techniques coupling HPLC with different spectroscopic detection methods have been developed. The use of on-line detection/identification systems allowing chemical screening of plant extracts not only for saponins but for a number of phytochemicals is a promising breakthrough in the determination and structural analyses of natural products. This detection/identification can be achieved by coupling LC-UV to mass spectrometry (LC-UV–MS) [80,143–145] or with nuclear magnetic resonance (LC-UV–NMR) [18,146,147].

In the LC-UV–MS mode three different interfaces are used most frequently. These include thermospray (LC–TSP-MS) [148], continuous flow FAB (LC–CF-FAB) [149], and electrospray (LC–ES-MS) technique [150]. The most suitable of these for phytochemical analysis is TSP, since this allows introduction of aqueous phase into MS system at a flow-rate compatible with that usually used in phytochemical analysis (1–2 ml/min). However, this interface allows satisfactory ionisation (NH_4OAc buffer, positive ion mode) of molecules within the mass range 200–800 mu, which means that only saponins having up to three sugars can be analysed satisfactorily [145]. For larger molecules with MW> 800 mu, CF-FAB or ES are the methods of choice [151]. Oleanolic acid glycosides from *Swartzia madagascarensis* Devaux in TSP mode showed clear MS molecular peaks only when they possessed up to three sugars. Much clearer spectra for three and four sugar-bearing saponins were obtained with CF-FAB and ES, but even though, the LC–MS–MS mode was necessary for full identification [18].

The LC–NMR method is not yet a widely accepted technique and many technical problems have still to be overcome including low sensitivity and the conditions for using non-deuterated solvents (MeCN, MeOH, H_2O) [18].

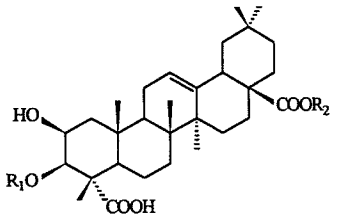
These methods, however, are still in the early stages of development and are generally regarded as pure research tools, without any major routine uses. However, for laboratories which can afford to purchase this extremely expensive equipment, the hyphenated HPLC techniques can be a powerful tool for efficient targeted isolation of new types of saponins with potential biological activities, replacing bioassay-guided search.

6. Capillary electrophoresis (CE) of saponins

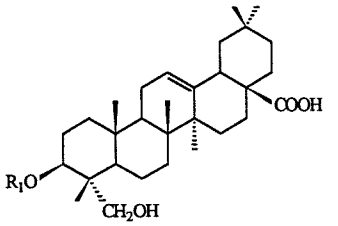
Capillary electrophoresis is an excellent analytical technique for the separation and quantitation of a broad range of phytochemicals. Millions of theoretical plates that can be obtained, rapid separation (1–30 min), minimal sample requirement (nl) and extremely low reagent consumption makes EC a very promising tool [152]. However, the minimal sample requirements for separation are in contrast with the high concentrations required for detection and this drastically limits the use of CE for saponin analysis [153].

The results of only a few trials on CE separation and determination of saponins have been published. Iwagami et al. [154] analysed ginseng saponins using the micellar electrokinetic capillary chromatography (MECC) mode with borate buffer at pH 7 with 25% ACN and 75 m*M* cholate as modifiers. The column 102 cm×50 μm (95 cm to detector) at 30 °C and 30 kV (+ve to −ve) were used. Detection was performed at 200 nm. Separation of triterpenoid in *Silene onites* was done by MECC with phosphate–borate buffer (pH 9.4), SDS and 5% MeOH. The 72 cm×50 μm (50 cm to detector) column heated to 50 °C and 20 kV (+ve to −ve) voltage were used for separation. Detection was performed at 240 nm. By this technique the more hydrophilic compounds had shorter migration times than the lipophilic ones and the elution pattern was very similar to that observed with reversed-phase separation [155]. The saponin concentration of herb mix I-tzu-teng was determined both with HPLC and CE [156]. In MECC technique with SDS and sodium cholate buffer, the complete separation of four anthraquinoids, five flavones, two carboxylic acids and saponins was obtained within 14 min, while for HPLC analysis 50 min were necessary.

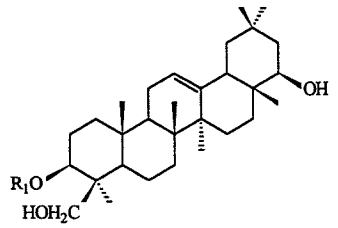
A number of medicagenic acid glycosides from alfalfa roots (Fig. 3) have been used for optimisation of CE separation [157]. After a number of experi-



1 R_1=Glcp R_2=[Xylp(1-4)Rhap(1-2)Arap]
2 R_1=Glcp R_2=Glcp
3 R_1=GlcAp R_2=[Xylp(1-4)Rhap(1-2)Arap]
6 R_1=Glcp R_2=H
7 R_1=[Glcp(1-2)Glcp] R_2=[Xylp(1-4)Rhap(1-2)Arap]



4 R_1=[Arap(1-2)Glcp(1-2)Arap]



5 R_1=[Rhap(1-2)Glcp(1-2)GlcAp]

Fig. 3. Chemical structures of alfalfa saponin standards used in HPLC and CE separation. Glcp, glucopyranosyl; GlcAp, glucuronopyranosyl; Arap, arabinopyranosyl; Xylp, xylopyranosyl; Rhap, rhamnopyranosyl.

ments with different temperatures (20–30 °C) and borate buffer concentrations (100–200 m*M*), the optimal conditions for separation of standard saponins were found to be 150 m*M* buffer concentration and room temperature. Under these conditions, complete separation was achieved within 7 min (Fig. 4). It was evident that glycosides having two free COOH groups eluted much later (one sugar saponin **6** and four sugars **3**) than the compounds with only one group free. Borate complexation at the OH groups of sugars and the mass effect due to different numbers of sugar units allowed separation of groups containing the same number of free COOH groups. The pattern of separation is substantially different from that obtained on reversed-phase HPLC [117]. Zanhic acid tridesmoside (not shown in the figures), which does not have free COOH eluted shortly after EOF, before any other compounds. There were also problems with registration of soyasaponin I, in spite of the fact it possesses COOH on the glucuronic acid molecule. Separation of the Sep-Pak C_{18} purified saponin mixture from alfalfa roots (Fig. 5) produced electrophoregraphs with a drift of baseline, indicating the need for further purification of the mixture prior to CE analysis.

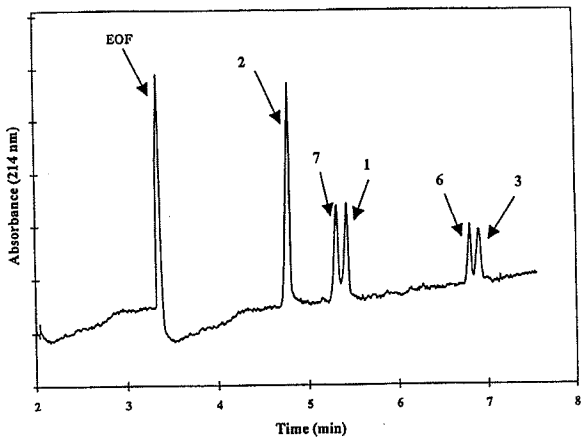


Fig. 4. CE separation of medicagenic acid (Ma) glycoside standards: EOF, internal standard; (**6**) 3 Glc Ma; (**2**) 3Glc,28Glc Ma; (**1**) 3Glc,28AraRhaXyl Ma; (**3**) 3GlcA,28AraRhaXyl Ma; (7) 3GlcGlc,28AraRhaXyl Ma.

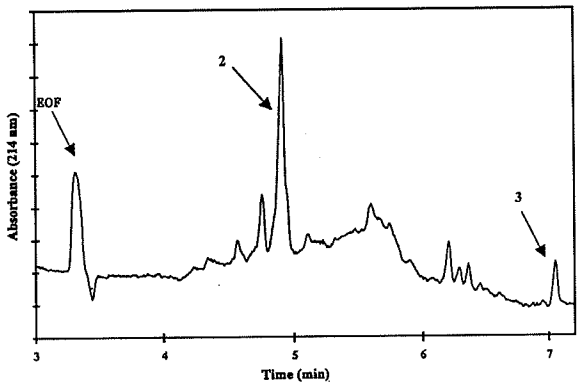


Fig. 5. CE separation of saponins from alfalfa roots: (2) 3Glc,28Glc Ma; (3) 3GlcA,28AraRhaXyl Ma.

The CE methods for saponin determination are also in the early stages of development but the data published so far give some hopes for future use of this technique in routine saponin analysis.

7. Conclusion

Chromatographic determination of saponins in plant material is still a challenge to the phytochemist. There is no one single method that can be recommended as routine procedure for analysis of complex saponin mixtures. None of these methods provides a fingerprint, which allows the quality to be monitored during the production of batches and to control properly the stability, which is required for registration of herbal medicinal products. Also none of them provides an adequate tool for the separation or preparative isolation of saponin components; a combination of several techniques is required to obtain single standard compounds.

All methods presented have advantages but also multiple limitations. Simple chromatographic methods (TLC, TLC–densitometry), if used properly by a well-trained person, can be of the same value as more sophisticated procedures including hyphenated techniques, which are not available in every laboratory due to the high cost. A good example is escin, the saponin mixture obtained from the seeds of *Aesculus hippocastanum* L, for which German Pharmacopoeia DAB 1997 recommends a colorimetric method.

High-performance liquid chromatography finds widest application. But since saponins possess a wide range of polarities and are lacking chromophores which allow UV detection, precise pre-column clean-up of the saponin fraction from the other components of the matrix is essential for this procedure. New types of more selective columns, allowing separation of saponins differing by just one terminal sugar are needed. Again work on escin can be a good example to follow; use of a Waters symmetry shield C_8 column can provide fingerprinting in just one chromatographic run [158].

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ANNEXURE 4

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Analysis of phenolic glycosides and saponins in *Primula elatior* and *Primula veris* (primula root) by liquid chromatography, evaporative light scattering detection and mass spectrometry

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Abstract

This paper describes the first liquid chromatographic method suitable for the simultaneous determination of bioactive compounds, saponins and phenolic glycosides, present in *Primula elatior* and *Primula veris*, including the NMR data of primulaverin and primeverin. Optimum separations were obtained with a Synergi 4 μm Fusion RP 80 Å column, using 0.025% TFA in water and 5% acetonitrile in methanol as mobile phase. Saponins were detected by evaporative light scattering detection (ELSD), whereas the phenolic glycosides were monitored by UV at 210 nm. The method was validated for repeatability ($\sigma_{rel} \leq 4.5\%$), precision (intra- and inter-day variation ≤5.0%), accuracy (recovery ≥97.1%) and sensitivity (LOD ≤22 ng (UV) and ≤38 ng (ELSD) on-column, respectively). LC–MS experiments in negative APCI mode allowed a final peak assignment. Both *Primula* species could easily be differentiated by their saponin pattern. The total saponin content was highest in *P. veris* roots (max. 14.9%), the aerial parts or *P. elatior* contained significantly less amounts; primeverin (0.64–1.42%) showed to be the most dominant phenolic glycoside.

Keywords: *Primula veris*; *Primula elatior*; ELSD; Mass Spectrometry; Saponins; Phenolic glycosides

1. Introduction

In the current, fourth edition of the European Pharmacopoeia two species are listed as source for primula (formerly primrose) root, *Primula elatior* L. Hill. (vernacular name: oxlip) and *Primula veris* (L.) (vernacular name: cowslip) [1]. Both plants, belonging to the Primulaceae family, are hardy perennial herbs native to Europe and temperate Asia; they can be distinguished by height (*P. elatior* is slightly taller) and coloration of flowers (oxlip has sulphurous-yellow, cowslip has pale-yellow flower heads). Hybridisation occurs quite often, which is no problem as the roots and rhizomes of both species are officinally used as Primulae radix [2].

The main indication for primula root is the treatment of respiratory tract problems, such as cough, asthma, bronchitis and catarrh. Responsible for these actions are secretolytic and secretomotoric triterpenoid saponins (Fig. 1), like priverosaponin B-22-acetate (**3**), primulasaponin I (**5**) and II (**4**), which are present in the plant material in rather high amounts up to 12% [3]. Safety and efficacy of primrose extracts rich in saponins have been demonstrated in a number of pharmacological studies, which showed potent anti-asthmatic, anti-inflammatory and anti-viral properties [4–7]. Phenolic glycosides (Fig. 1), mainly primulaverin (**1**) and primeverin (**2**), are characteristic compounds for the genus *Primula*. They degrade during storage in presence of the enzyme primverase, resulting in the typical fragrance of the drug [2]. Thus, they not only serve as marker compounds but also as indicators of the age of the plant material.

Analytical methods for the analysis of primula root are quite rare, most likely because the saponins possess no chromophore and therefore are difficult to detect by UV. Besides determinations by TLC [8] and spectrophotometry [9,10], only one HPLC method for the analysis of primula saponins has been reported [11]. Being published nearly 20 years ago, the latter is still the most recent analytical report on Primulae radix, but it does not enable the determination of all relevant saponins or the simultaneous analysis of saponins and phenolic glycosides. In addition the method was not validated. Thus, the study presented herein aimed to improve the established HPLC methodology in respect to sensitivity, number of analytes, validity and ease of operation. By means of ELSD (which previously showed to be ideal for the analysis of low absorbing compounds; [12]) and MS, the

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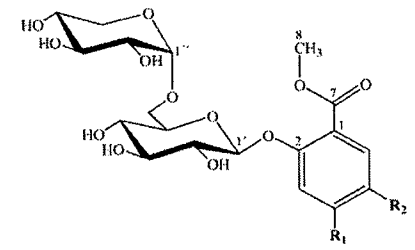
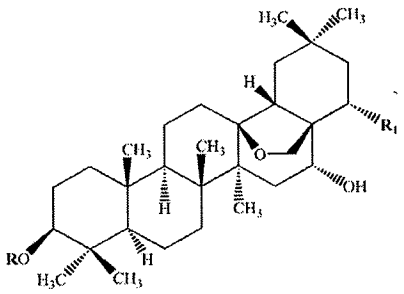
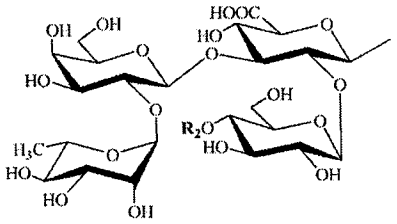


R_1	R_2	
-H	-O-Me	**1** (Primulaverin)
-O-Me	-H	**2** (Primeverin)



R:



R_1	R_2	
-O-Ac	-H	**3** (Priverosaponin B 22-acetate)
-H	- β-D-Xyl	**4** (Primulasaponin II)
-H	-H	**5** (Primulasaponin I)

Fig. 1. Structures of compounds **1–5**.

distribution of phenolic glycosides and saponins in *P. veris* and *P. elatior* and different plant parts was investigated.

2. Experimental

2.1. Materials

Compounds **1–5** were isolated by chromatographic techniques from commercial plant material. Identity and purity (>95%) of the isolated compounds were confirmed by TLC, HPLC, MS, 1D- and 2D-NMR experiments. A final structural assignment of **3–5** was achieved by a comparison of the obtained NMR shifts with literature values [13–15]; for compounds **1** and **2** no NMR data has been published (see Section 2.2). Besides **1–5** another major compound (**6**) was present in all root samples analyzed. It shows similar properties like the aforementioned saponins (e.g. mass range and polarity, no UV absorption), with a molecular weight of *m/z* 1178; thus, it was assumed to be a saponin. A saponin with this molecular mass has not been reported from primula so far and its isolation and structural elucidation is currently in progress.

Plant material (*P. veris*: PV-1 to PV-3; *P. elatior*: PE-1 to PE-3) was collected between May and June 2004 in several locations in Austria (Wies: PE-1, PV-1; Innsbruck: PE-2, PE-3, Landeck: PV-3) and Germany (Merseburg: PV-2) and authenticated by Prof. Dr. C. Zidorn (Institute of Pharmacy, University of Innsbruck, Austria). Voucher specimens of all samples are deposited at the same institution.

Solvents (acetonitrile, methanol) were of HPLC grade and purchased from Merck (Darmstadt, Germany); water was purified by nanopure filtration prior to HPLC use.

2.2. NMR data of primulaverin and primeverin

NMR spectra of primulaverin (**1**) and primeverin (**2**) were recorded on a Bruker-AM-300 spectrometer at 300 MHz (^{1}H) and 75 MHz (^{13}C). Both substances were dissolved in DMSO-d_6 and spectral data were referenced to solvent residual signals at $\delta_H = 2.50$ ppm and $\delta_C = 39.5$ ppm.

1: ^{1}H NMR: *methoxy salicylic acid ester moiety*; δ 7.32 (1H, d, $J = 9.0$ Hz, H-3), 7.13 (1H, d, $J = 3.0$ Hz, H-6), 7.10 (1H, dd, $J = 9.0$, 3.0 Hz, H-4), 3.80 (3H, s, H-8), 3.75 (3H, s, H-9), *primverosyl moiety*: δ 4.70 (1H, d, $J = 7.5$ Hz, H-1′), 4.19 (1H, d, $J = 7.5$ Hz, H-1″), 3.97 (1H, brd, $J = 11.0$ Hz, H-6$_a$′), 3.68 (1H, dd, $J = 11.0$, 5.5 Hz, H-5$_a$″), 3.56 (1H, dd, $J = 11.0$, 6.5 Hz, H-6$_b$′), 3.49 (1H, t, $J = 6.5$ Hz, H-5′), 3.26 (3H, m*, H-2′,3′,4″), 3.17 (1H, d, $J = 5.0$ Hz, H-4′), 3.06 (1H, dd, $J = 8.0$, 4.5 Hz, H-3″), 3.00 (1H, dd, $J = 7.5$, 4.5 Hz, H-2″), 2.95 (1H, d, $J = 11.0$ Hz, H-5$_b$″); ^{13}C NMR: *methoxy salicylic acid ester moiety*; δ 166.3 (C-7), 153.6 (C-5), 150.2 (C-2), 122.3 (C-1), 119.0 (C-4), 118.7 (C-3), 113.9 (C-6), 55.3 (C-9), 51.7 (C-8), *primverosyl moiety*: δ 103.6 (C-1″), 101.7 (C-1′), 76.1 (C-3″), 76.0 (C-3′), 75.6 (C-

5′), 73.0 (C-2′, 2″), 69.4 (C-4′), 69.3 (C-4″), 68.0 (C-6′), 65.3 (C-5″).

2: ^{1}H NMR: *methoxy salicylic acid ester moiety*; δ 7.67 (1H, d, J = 9.0 Hz, H-6), 6.81 (1H, d, J = 2.0 Hz, H-3), 6.66 (1H, dd, J = 9.0, 2.0 Hz, H-5), 3.81 (3H, s, H-9), 3.76 (3H, s, H-8), *primverosyl moiety*: δ 4.91 (1H, d, J = 7.5 Hz, H-1′), 4.15 (1H, d, J = 7.5 Hz, H-1″), 3.95 (1H, d, J = 10.0 Hz, H-6$_a$′), 3.67 (1H, dd, J = 11.0, 5.3 Hz, H-5$_a$″), 3.60 (2H, m*, H-5′, H-6$_b$′), 3.30 (2H, m*, H-2′, H-3′), 3.25 (1H, m, H-4″), 3.20 (1H, m, H-4′), 3.07 (1H, br t, J = 8.5 Hz, H-3″), 3.00 (1H, d, J = 11.0 Hz, H-5$_b$″), 2.95 (1H, br t, J = 6.0 Hz, H-2″); ^{13}C NMR: *methoxy salicylic acid ester moiety*; δ 165.7 (C-7), 163.6 (C-4), 158.5 (C-2), 132.4 (C-6), 112.7 (C-1), 107.7 (C-5), 102.1 (C-3), 55.5 (C-9), 51.6 (C-8), *primverosyl moiety*: δ 104.1 (C-1″), 100.9 (C-1′), 76.5 (C-3″), 76.2 (C-3′), 75.7 (C-5′), 73.3 (C-2″), 73.2 (C-2′), 69.6 (C-4′), 69.5 (C-4″), 65.6 (C-5″).

*Overlapped.

2.3. Preparation of samples

Finely powdered plant material (0.125 g of roots, 0.300 g of flowers and leaves) was extracted five-fold by sonication for 10 min with 5.00 ml 50% (v/v) aqueous methanol. After centrifugation (10 min at 3000 rpm) the supernatants were combined in a 25 ml volumetric flask and finally filled up with extraction solvent. Each sample solution was filtered through a 0.45 μm cellulose acetate membrane filter (Sartorius, Göttingen, Germany) and injected in triplicate.

2.4. Calibration

Three milligrams of the phenolic glycosides (**1**, **2**) and 2.5 mg of each saponin (**3**–**5**) were weighted out into one 5 ml volumetric flask and dissolved in methanol (stock solution). Further calibration levels were prepared by diluting this solution 1:1 with methanol (injected concentration ranges: 18.8–600 μg ml^{-1} (**1**, **2**) and 15.6–500 μg ml^{-1} (**3**–**5**)). Because of their similar MS pattern the not yet identified compound **6** was quantified based on the calibration data of **3**. Within this range of concentrations the detector response was linear; see Table 1 for combined calibration data. The calibration curves for **1** and **2** were obtained by linear regression based on peak area recorded at 210 nm. The response of an ELS detector follows an exponential relationship, thus plotting log of peak area versus log of concentration will provide a linear response [16]. All data were recorded and edited by ChemStation software (Agilent, Waldbronn, Germany).

Table 1
Calibration data for compounds **1**–**5**

Compound	Detection	Regression equation	R^2	LOD (ng on column)
1	UV – 210 nm	$y = 16.381X - 21.475$	0.9999	22
2	UV – 210 nm	$y = 14.940X - 29.741$	0.9999	23
3	ELS	$y = 1.561X + 2.048$	0.9985	38
4	ELS	$y = 1.613X + 1.917$	0.9993	34
5	ELS	$y = 1.569X + 2.023$	0.9990	33

y: represents the peak area (mAu × s), X: the amount of compound (μg ml^{-1}), R^2: correlation coefficient, LOD: limit of detection. For ELS-detection: y: log (peak area), X: log (concentration in μg ml^{-1}).

2.5. Analytical method

Primula samples were analyzed on a Hewlett Packard 1090 series liquid chromatograph (Agilent Technologies, Waldbronn, Germany), equipped with column heater, diode array detector and autosampler, coupled to an Alltech 2000 evaporative scattering light detector (Deerfield, IL, USA). LC separations were achieved using a 150 mm × 4.6 mm Synergi 4 μm Fusion RP 80 Å column (Phenomenex, Torrance, CA, USA) at a temperature of 50 °C. The mobile phase comprised water containing 0.025% TFA (v/v; A) and methanol containing 5% acetonitrile (v/v; B). Elution was performed using the following gradient: in 25 min from 85A/15B to 60A/40B, in 3 min to 50A/50B, in further 20 min to 5A/95B and held at that composition for 7 min (analysis time: 55 min); each separation was followed by an equilibration period of 10 min. The UV detector was set to a wavelength of 210 nm, the flow rate to 1.0 ml min^{-1} and the injection volume to 10 μl, respectively. The ELSD parameters were adjusted to a nebulizer temperature of 97.0 °C, a nitrogen-flow rate of 2.1 l min^{-1} and gain 8.

LC–atmospheric pressure chemical ionisation mass spectrometry (LC–APCI-MS) studies were performed on an Esquire 3000 plus ion-trap mass spectrometer (Bruker Daltronics, Bremen, Germany) and a Hewlett Packard 1100 series liquid chromatograph (Agilent Technologies, Waldbronn, Germany). Best results were obtained in negative APCI mode with corona capillary, nebulizer, dry-gas, APCI temperature, dry temperature and solvent split ratio set to −10,000 nA, 40.0 psi (nitrogen), 4.0 l min^{-1} (nitrogen), 400 °C, 300 °C and 1:3, respectively. The scan range was adjusted to m/z 200–1400 and the target mass set to m/z 750. The same LC conditions as described above were used, except mobile phase that was changed to 0.1% acetic acid (v/v; A) and acetonitrile (B) to obtain a better ionization of the analyzed compounds. MS-data were recorded and processed using Bruker Daltronics data analysis software, version 3.0.

2.6. Method validation

The developed HPLC-UV-ELSD method was validated for linearity (see Section 2.4), peak purity, limit of detection, accuracy, precision and repeatability.

Peak purity was evaluated based on the DAD-data of **1** and **2** as well as the MS-data of all compounds of interest (Fig. 3). Limit of detection was determined by serial dilution of a standard solution containing **1**–**5** (Table 1). To evaluate the methods accuracy, oxlip root powder (sample PE-1, 0.125 g) was spiked with three concentration levels of known amounts of the phenolic glycoside **2** and the saponin **5**. The material was then extracted and assayed under optimized conditions to determine respective recovery rates.

Intra- and inter-day precision of the method were evaluated by assaying sample PV-1 five-fold on one day, repeating the same procedure on two more days; for detailed results see Table 2. Repeatability was confirmed by investigating unifor-

Table 2
Intra- and inter-day precision if one sample (PV-1, root) is analysed under optimized conditions

Compound	Intra-day ($n=5$)			Inter-day ($n=3$)
	Day 1	Day 2	Day 3	
2	0.94 (2.40)	0.95 (4.31)	0.94 (4.36)	0.94 (1.53)
3	1.65 (3.34)	1.65 (2.87)	1.71 (4.98)	1.67 (2.09)
5	5.52 (4.51)	5.51 (3.76)	5.53 (2.94)	5.52 (0.16)
6	3.71 (3.17)	3.68 (2.10)	3.64 (1.86)	3.68 (0.97)

Values in % (mg 100 mg^{-1}); RSD are given in parentheses.

mity of retention times and RSDs (σ_{rel}) for triplicate injections (Tables 3 and 4).

3. Results and discussion

The analysis of a standard mixture of **1–5**, under optimized conditions, is shown in Fig. 2A. All five compounds are readily assignable with ELS-detection, whereas at 210 nm only the phenolic glycosides **1** and **2** appear as distinct signals. To obtain this separation and peak shape, the mobile phase had to be acidic, otherwise the saponins would elute as broad, unsymmetrical signals. Trifluoroacetic acid (TFA) showed to be ideal, as it resulted in a minimal baseline drift at 210 nm. For the analysis of real-life samples (Fig. 2B) the methodology had to be further refined, as other, interfering compounds are present in the plant material (e.g. large amounts of flavonoids in the flowers). Phenolic glycosides and flavonoids are of similar polarity, and **1** and **2** co-eluted with some flavonoids if acetonitrile was used as organic mobile phase. By screening several types of stationary phases with different particle sizes, it was revealed that only one particular column (Synergi Fusion-RP from Phenomenex) enabled their separation. For an optimum separation 0.025% TFA and

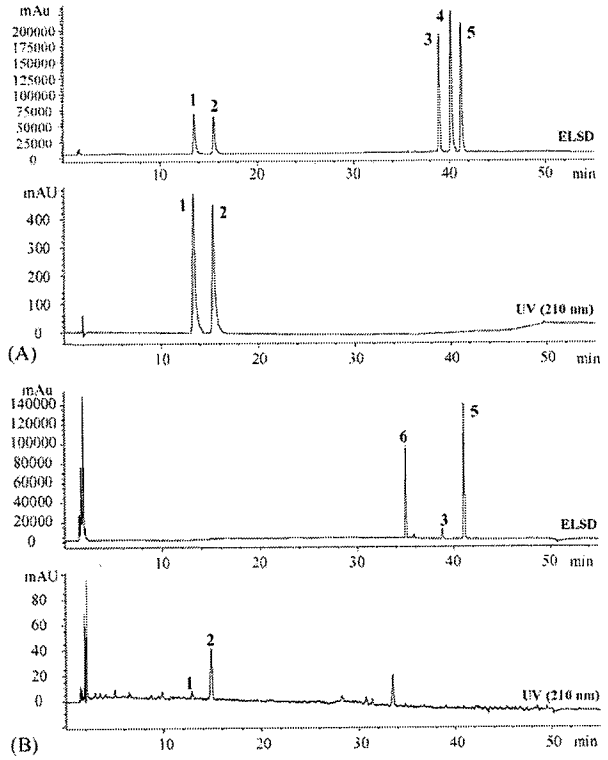


Fig. 2. Separation of a standard mixture of **1–5** (A) and a primula root extract (PV-2; B) under optimized HPLC conditions [column: Synergi Fusion RP, 4 μm, 150 mm × 4.6 mm; mobile phase: 0.025% TFA in water (A), methanol containing 5% acetonitrile (B), gradient: $t=0$ min: 85A/15B, $t=25$ min: 60A/40B, $t=28$ min: 50A/50B, $t=48$ min: 5A/95B, stoptime = 55 min; flow rate: 1.0 ml min^{-1}; detection: 210 nm and ELSD, injected sample volume: 10 μl, temperature: 50 °C; ELSD conditions: nitrogen flow: 2.1 ml min^{-1}, gain: 8, temperature: 97.0 °C]; assignment of **1–5** according to Fig. 1; **6** is an unknown compound.

Table 3
Analysis of **1–6** in different parts of *Primula veris*

Compound	PV-1			PV-2			PV-3		
	Roots	Flowers	Leaves	Roots	Flowers	Leaves	Roots	Flowers	Leaves
1	–	–	–	0.06 (0.69)	–	–	0.09 (0.95)	–	–
2	0.70 (0.95)	–	–	1.42 (0.57)	–	–	0.64 (2.20)	–	–
3	1.67 (3.83)	–	–	1.87 (1.61)	–	–	1.39 (3.32)	–	–
4	0.32 (1.71)	–	–	0.23 (4.03)	–	–	–	–	–
5	5.71 (2.44)	0.28 (2.72)	2.09 (2.79)	8.26 (2.08)	0.20 (2.64)	3.30 (1.96)	7.14 (0.89)	0.22 (4.41)	1.07 (1.96)
6	3.81 (2.11)	–	–	4.56 (1.07)	–	0.18 (3.10)	5.30 (3.39)	0.07 (3.47)	–

Values in % (mg 100 mg^{-1}); RSD are given in parentheses ($n=3$). (–) Not detectable.

Table 4
Analysis of **1–6** in different parts of *Primula elatior*

Compound	PE-1			PE-2			PE-3		
	Roots	Flowers	Leaves	Roots	Flowers	Leaves	Roots	Flowers	Leaves
1	–	–	–	0.11 (1.14)	–	–	0.08 (1.20)	–	–
2	–	–	–	0.05 (1.74)	–	–	0.09 (0.94)	–	–
5	3.45 (3.74)	0.41 (4.51)	1.74 (0.78)	5.94 (1.33)	0.45 (1.89)	1.09 (1.03)	5.60 (2.94)	0.39 (4.51)	3.95 (0.69)
6	–	–	0.17 (3.42)	–	–	–	–	–	0.14 (2.35)

Values in % (mg 100 mg^{-1}); RSD are given in parentheses ($n=3$). (–) Not detectable.

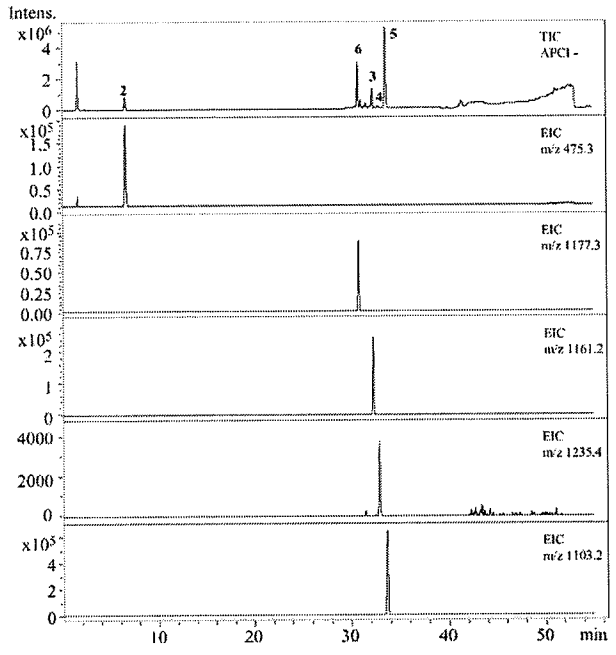


Fig. 3. LC/MS chromatogram of a *P. veris* root extract (PV-1) under LC-conditions as for Fig. 2, except mobile phase 0.1% acetic acid (A), acetonitrile (B); MS-conditions: APCI neg. mode, nebulizer: 40 psi, dry gas: 4.0 ml min^{-1}, dry temperature: 300 °C, APCI temperature: 400 °C, corona: −10,000 nA, solvent split ratio 1:3; assignment of **2–5** according to Fig. 1; **6** is an unknown compound.

methanol were used as mobile phase. Addition of 5% acetonitrile to the methanol and a raised separation temperature (50 °C) further improved the result in respect to required separation time and peak symmetry. By adding other modifiers (MBE, THF), or by using a buffer (ammonium acetate), the separation could not be improved. Based on the initial gradient settings (85A/15B) and as suggested by the instruments software, the ELS detector was adjusted to a nebulizer temperature of 97.0 °C and a nitrogen flow-rate of 2.1 l min^{-1}, respectively.

In addition to assign peaks based on their retention times, UV-spectra (for phenolic glycosides), and spiking the extracts with reference compounds, LC–MS experiments were performed to finally assure peak identity. All five standard compounds were directly assignable in the plant extracts in negative APCI mode (Fig. 3). To achieve a good response and ionisation, the solvent composition had to be changed to 0.1% acetic acid and acetonitrile. These solvents did not result in the optimum separation (see above), while in EIC (extracted ion current) mode all compounds could be clearly identified in the chromatograms.

To confirm our methods validity several analytical and statistical parameters were determined. Calibration data of **1–5** (Table 1) indicated linearity of the detector signal within the concentration range injected ($R^2 \geq 0.9985$) and a limit of detection (*S/N* ratio of 3; determined by serial dilution) of 22 ng (**1**; UV-detection) and 38 ng on-column (**3**; ELSD) or less. Peak purity was confirmed by two methods: for UV-absorbing compounds the "peak purity evaluation" option of the Chemstation software was utilized (threshold value set to 975), for saponin compounds the obtained MS-information served as evidence. Based on these data all relevant peaks in the sample chromatograms were found to be free of impurities or co-elutions. Accuracy was determined by spiking one sample with three different concentrations of primeverin (**2**) and primulasaponin I (**5**) (representing 80, 100 and 120% of the expected value); the compounds served as representatives for the two classes of compounds investigated. The spiked samples were extracted and analyzed under optimized conditions and recovery rates close to 100% were obtained at all three levels. For the phenolic glycoside **2** they were found to be between 99.0 and 99.7%, for the saponin **5** from 97.1 to 100.2%.

All standards and samples were injected in triplicate. SDs below 5.0% (max. $\sigma_{rel} = 4.51\%$ for **5** in PE-3) confirmed repeatability of the developed assay. Intra- and inter-day variation of the method were determined and showed to be lower than 5.0% as well, with a maximum RSD of 4.98% reached at day 3 for compound **3** (Table 2).

Finally, several specimens of *Primula* plant material (*P. veris*: PV-1 to PV-3; *P. elatior*: PE-1 to PE-3; all samples divided into different plant parts) were analyzed (Fig. 4). All three classes of compounds could be separated, i.e. phenolic glycosides eluted between 13 and 16 min, flavonoids between 16 and 27 min, and saponins from 35 to 42 min. Mean values of the individual compounds and RSDs ($n = 3$) were calculated and are shown in Tables 3 and 4. Saponin **5** was most dominant and present in all samples investigated, with the highest content in roots (e.g. 8.26%, PV-2) and lowest concentration in flowers (e.g. 0.20 %, PV-2). Only in cowslip (*P. veris*) roots several of the other saponins (**3**, **4** and **6**) were detectable, a differentiation of root material from species being therefore possible. The other plant parts (*P. elatior* leaves; *P. veris* leaves/flowers) contained only small amounts (less than 0.2%) of the compound **6** additionally. The total saponin content in roots ranged between 3.45 (PE-1) and 14.92% (PV-2), in flowers from 0.20 (PV-2) to 0.45% (PE-2) and in leaves from 1.07 (PV-3) to 4.09% (PE-3); the quantitative results for saponins in root material are in good agreement to the literature [3,11]. According to literature, phenolic glycosides (**1**, **2**) are only present in the underground parts of *Primula* ssp. [2]. The obtained results confirmed this statement, with

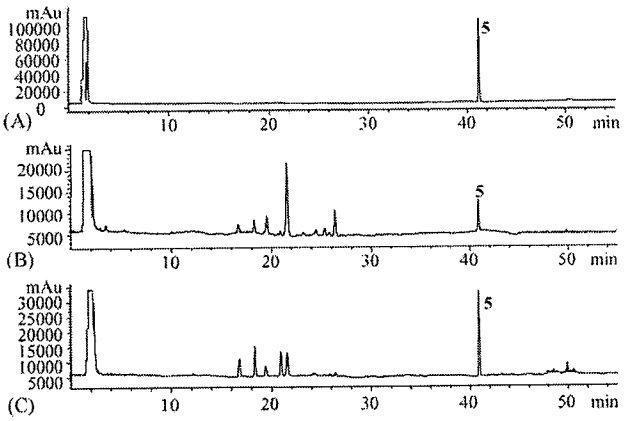


Fig. 4. Determination of saponins in different plant parts of sample PE-2 (A: roots, B: flowers, C: leaves) by ELSD; for optimized conditions see Fig. 2.

primeverin (**2**) being dominant in most of the samples analyzed (0.64–1.42%). Only in one *P. elatior* sample compound **1** was found in slightly higher amounts than **2** (0.11 versus 0.05% in PE-2, respectively). The phenolic glycoside content appeared to be lower in *P. elatior* in general, with one sample (PE-1) containing no **1** or **2** at all.

4. Conclusions

Considering that *P. veris* and *P. elatior* are two important and widely used medicinal plants in Europe, their differentiation and quality assessment is of great importance. Thus, the method presented herein is aimed to achieve both goals for the first time. In fact, it allows the exact quantitative estimation of all (pharmacologically) relevant compounds (phenolic glycosides and saponins), and based on the saponin pattern, enables the differentiation of both species (root material). Thus, the newly developed method should be useful for a wide range of possible commercial, agricultural and scientific applications.

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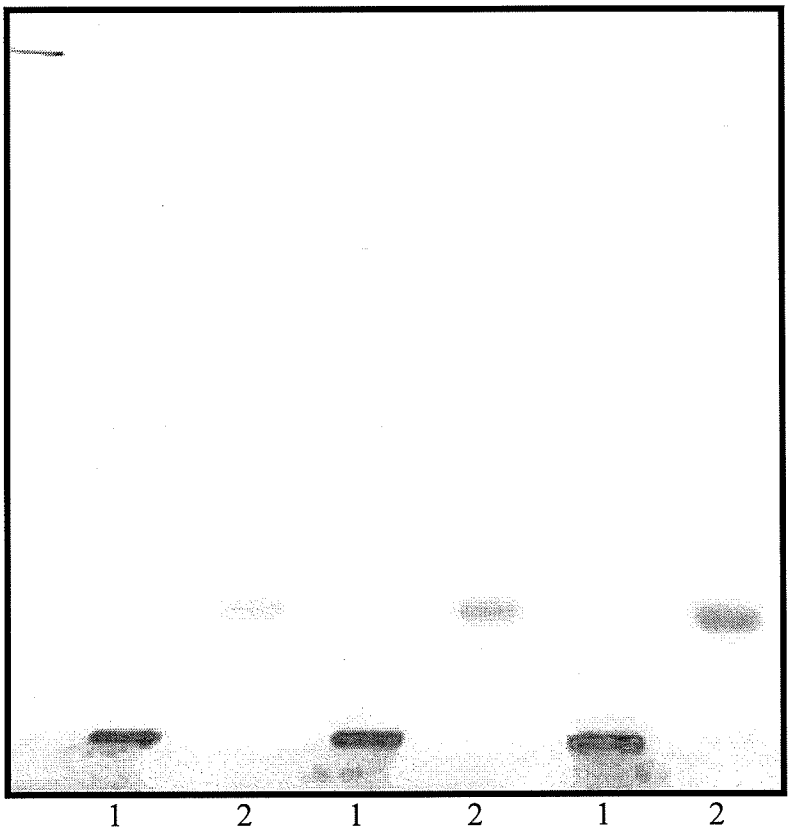
ANNEXURE 5

ANNEXURE-IX

TLC COMPARISON BETWEEN *S. TRIFOLIATUS* EXTRACT OF THE PRESENT INVENTION AND HEDERAGENIN

Fig.1: TLC using less polar mobile phase



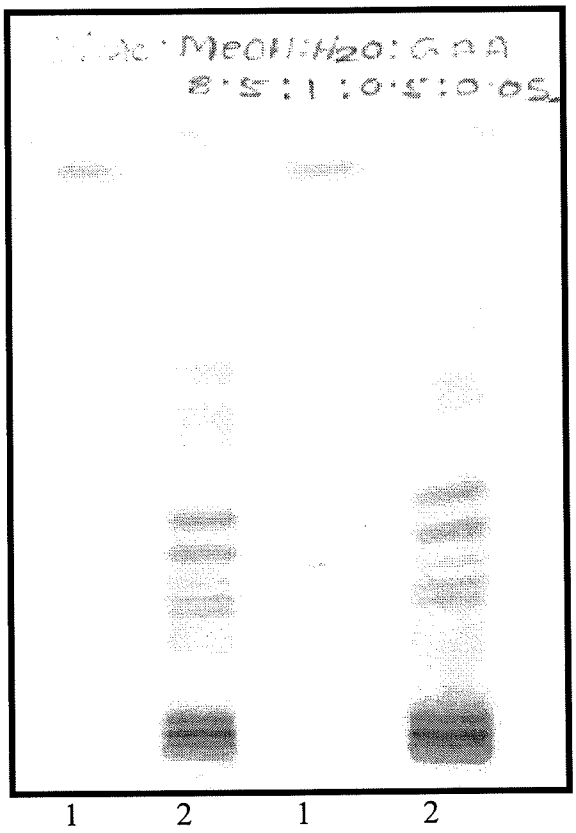
TLC CONDITION

Sample 1 : *S. trifoliatus* aqueous extract of the present invention
Sample 2 : Hederagenin standard sample [from *Extrasynthese*]

Sample Concentration

Sample 1	: 20 mg/ml in MeOH:Water (1:1)
Sample 2	: 5 mg/ml in $CHCl_3$:MeOH (70:30)
TLC Plate	: Silica gel $60F_{254}$ Merck
TLC Applicator	: CAMAG Linomat IV
Volume applied	: 2µl, 4µl & 6µl
Solvent System	: Ethyl acetate : Hexane (60:40)
Spray Reagent	: Vanillin Sulphuric acid
Visualization	: After heating at 110°C

Fig. 2: TLC using high polar mobile phase



TLC CONDITION

Sample 1 : Hederagenin standard sample [from *Extrasynthese*]
Sample 2 : *S.trifoliatus* aqueous extract

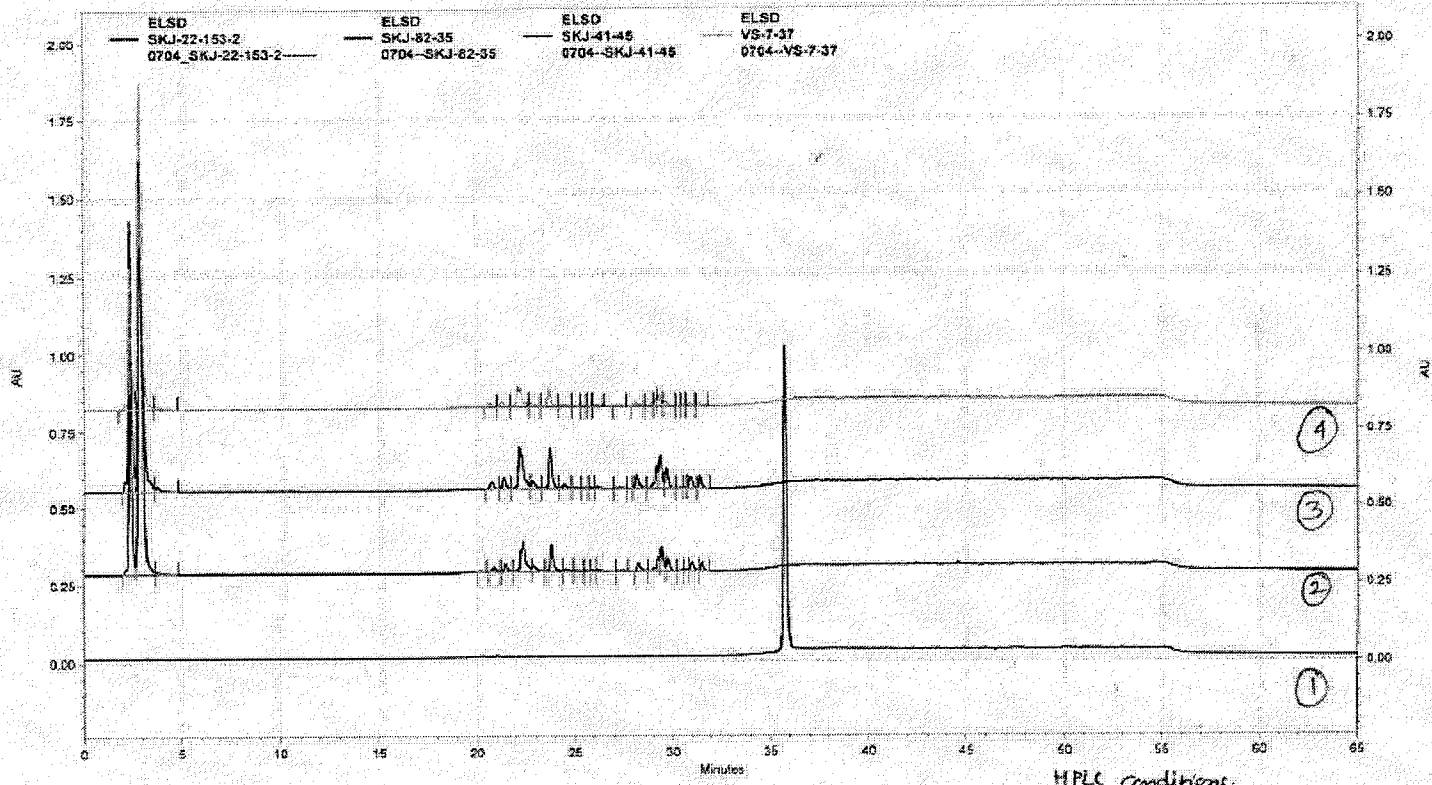
Sample Concentration

Sample 1	: 5 mg/ml in $CHCl_3$:MeOH (70:30)
Sample 2	: 20 mg/ml in MeOH:Water (1:1)
TLC Applicator	: CAMAG Linomat IV
TLC Plate	: Silica gel $60F_{254}$ Merck
Volume applied	: 2µl & 4µl
Solvent System	: EtOAc : MeOH : Water : Glacial acetic acid (8.5 : 1 : 0.5 : 0.05)
Spray Reagent	: Vanillin Sulphuric acid
Visualization	: After heating at 110°C

Conclusion: Free hederagenin is not present in the *S. trifoliatus* aqueous extract of the present invention

ANNEXURE 6

Annexure – X: HPLC COMPARISON OF LLL-3042 AND HEDERAGENIN
Sample no. 1: Hederagenin; Samples 2-4: Extracts of *S. trifoliatus* of the present invention



HPLC conditions.
Buffer: 0.1% Formic acid
Mobile phase: Buffer + Acetonitrile
gradient method
Column: Cosmosil C18 250x4.6mm
Detector: ELSD Temp 50°C, Gain-9
Sample: 1mg/ml in water
Hederagenin: 1mg/ml (THF/MeOH 50/50 v/v)